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The effects of stressors during early life on hippocampal neurogenesis and microglial activation in the male and female brain

Thesis presented by

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University College Cork

Department of Anatomy and Neuroscience

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Supervisors: Prof. Yvonne Nolan, Dr Olivia O'Leary

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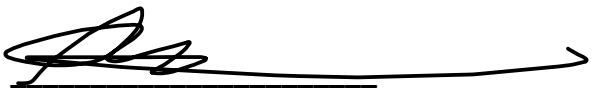
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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.



Andrew J. McGovern

September 2020

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Author Contributions

The author conducted all work presented within this thesis independently, with the exception of the following contributions:

- Dr Siobhain O'Mahony carried out the maternal separation procedure on animals used in one part of this study.
- Dr Cara Hueston injected animals with saline and lipopolysaccharide and completed the perfusion of animals used in the first part of this study.
- Dr Erin Harris conducted the juvenile stress paradigm in male and female rats as well as the tissue collection from animals used in the second half of this study.

Abstract

Stress during critical periods of brain development and maturation such as adolescence is associated with an increased risk of developing stress-related psychiatric disorders which are more common in women than men. Early life stress such as maternal separation (MS), juvenile stress (JS) and inflammatory insults like lipopolysaccharide (LPS), have been found to induce anxiety and depressive-like behaviours and decrease adult hippocampal neurogenesis in rodents. However, the effects of early life stress on adult hippocampal neurogenesis and associated function have been mostly assessed in male rodents. The impact of early life stress on microglia, which are involved in the regulation of adult hippocampal neurogenesis and dendritic remodelling, has also been predominantly examined in male rodents. Thus, in this study we assessed adult hippocampal neurogenesis and hippocampal microglia following LPS administration in MS juvenile female Sprague-Dawley rats and following JS in male and female Sprague-Dawley rats in adulthood.

MS increased the number of newly born hippocampal neurons in the ventral hippocampus, reduced the dendritic complexity of newly born neurons in the whole hippocampus and increased the soma size of microglia, indicating activation. LPS reduced newly born hippocampal dendritic complexity and increased the number of microglia in the dorsal hippocampus. Conversely, LPS administration in MS rats reduced the number of microglia in the dorsal hippocampus and MS attenuated microglial activation in response to LPS. LPS administration in MS increased dendritic complexity in the granule cell layer (GCL) and further reduced dendritic complexity in the ventral but not dorsal hippocampus of juvenile female rats. JS did not affect

hippocampal neurogenesis in adult male or female rats but reduced the cell soma size of microglia in the GCL in the dorsal hippocampus of females. We observed significant sex differences in adult rats; females had fewer newly born neurons with less dendritic complexity in the dorsal hippocampus than males. There were also fewer microglia in the molecular layer (ML) of the hippocampus in adult female than male rats. Together the data here shows that the effect of early life stressors differentially affects hippocampal neurogenesis and hippocampal microglia dependent on age, sex and subregion of the hippocampus analysed.

Abbreviations

BDNF	Brain Derived Neural Growth Factor
CA	Cornu Ammonis
DAB	3,3'-Diaminobenzidine
DCX	Doublecortin
DG	Dentate Gyrus
EC	Entorhinal Cortex
GCL	Granule Cell Layer
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Adrenal
IBA-1	Ionized Calcium Binding Adaptor Molecule 1
IL	Interleukin
JS	Juvenile Stress
LPS	Lipopolysaccharide
ML	Molecular Layer
MS	Maternal Separation
NPC	Neural Precursor Cell
NS	Non-Separated
NSC	Neural Stem Cell

PND	Postnatal Day
Poly(I:C)	Polyinosinic:polycytidylic acid
SAL	Saline
SGZ	Subgranular Zone
SUB	Subiculum
TLR	Toll-Like Receptor
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular Endothelial Growth Factor

1. General introduction

Stress is associated with many physical and emotional consequences such as the development of psychiatric disorders including depression or anxiety (Davis *et al.* 2017). The World Health Organisation has found that over 322 million people worldwide suffer from depression and 264 million suffer from anxiety with most of these cases being co-morbid (World Health 2017). Depression is also the leading cause of disability worldwide (World Health 2017).

The pathophysiology of a stress-related psychiatric disorder is as unique as an individual's genetics and life experience. The causes of depression can be in response to an acute stressor, chronic stressor, genetic predisposition or substance abuse (Silberg *et al.* 1999; Kuria *et al.* 2012). Not only this, but the age at which a traumatic event is experienced can impact the development of the stress-related psychiatric disorders (Copeland *et al.* 2018). The sex of an individual can also influence the likelihood and severity of stress-related psychiatric disorders, with significantly more women diagnosed with stress-related psychiatric disorders (Kuehner 2017). For example, women who experience early life trauma are 3.84 times are more likely to develop a mental health disorder in adulthood (Martins-Monteverde *et al.* 2019).

It is well established that the development of stress-related psychiatric disorders, such as major depressive disorder or major anxiety disorder can be caused by exposure to multiple significant stressors (Kendler *et al.* 2001). An individual may experience multiple stresses and exposures to inflammatory insults across the lifespan. This concept of allostatic loading was first introduced by McEwen and Steller in 1993, who described an allostatic load as an accumulation of stressors. These

stressors include chronic stressors (environmental or social), acute stressors (death, divorce or abuse) or physiological stressors (Inflammation (illness), metabolic or circadian disruptions) (Danese and McEwen 2012). The immune system and stress response are tightly interlinked (Marketon and Glaser 2008). Acute and chronic stress leads to an altered immune profile and immune system activity alters the neuroendocrine stress profile (Padgett and Glaser 2003). Stressors don't act in a singular manner, but in fact against both an individual's endocrinal and immunological profiles (Parker and Douglas 2010; del Rey and Besedovsky 2017). Stressors can also have different effects due to genetics and/or prior experiences (individual differences) (Ebner and Singewald 2017). Current research aims to understand the long-term effects of stressors, how multiple stressors may interact and how individual differences (such as sex differences) interplay with stress in animals and humans (Rubinow and Schmidt 2019). It is important to understand the immediate effect of stress but also the long term adaptations to stress, which may increase our insight into stress-related psychiatric disorders (Osório *et al.* 2017).

A critical period is a period in ongoing development when a system in the body is especially sensitive to extrinsic stimuli (McCarthy *et al.* 2018; Nelson Iii and Gabard-Durnam 2020). Critical periods encompass the prenatal, postnatal and adolescent (juvenile and post pubertal) periods prior to adulthood, where high rates of ontogenetic change are evident (van den Berg *et al.* 2014; Selemon and Zecevic 2015). Stress during critical periods may interrupt development, such as the brains neural and immunological systems (VanRyzin *et al.* 2018). Although stressors such as early life adversity or early life inflammation are primarily driven via different mechanisms (hypothalamic-pituitary-adrenal (HPA) axis and immunological) they

both induce microglial activation and detrimental effects to the cellular process of hippocampal neurogenesis (which will be discussed further in this thesis) (Green and Nolan 2014; Lajud and Torner 2015; Roque *et al.* 2016; Bunea *et al.* 2017; Nettis *et al.* 2019).

Clinical studies have established that stressors such as childhood neglect, physical or sexual abuse and illness during critical periods leads to a significant increase in the likelihood of developing a stress-related psychiatric disorder later in life (Negele *et al.* 2015). Children are more vulnerable to the disruptive effects of stress than adults, prior to adulthood the body is both behaviourally and molecularly primed to react to stress (Romeo 2015). In humans, stressors during adolescence can increase the risk of developing major depressive disorder, anxiety, heart disease, post-traumatic stress disorder and chronic fatigue syndrome (Pelcovitz *et al.* 1994; Kendler *et al.* 2001; Low *et al.* 2009; McLaughlin and Hatzenbuehler 2009; Jason *et al.* 2014).

The disruption of normal brain development via stress by altered endocrine or immunological profiles may initially be interpreted as detrimental, but the developmental changes (and stress response itself) function to alter the animal's behaviour towards an appropriate pro-survival behaviour (Monaghan and Haussmann 2015). Our immediate reactions to stress are an immediate survival behaviour whilst the long-term effects of stress are an altered long-term survival behaviour (Ellis and Del Giudice 2019). An introduction to stress early in life may act as a signal to alternatively develop our brain for a life of coping and importantly surviving with stress (Gluckman *et al.* 2007; Agorastos *et al.* 2019).

1.1 The hippocampus

1.1.1 The hippocampus's structure

The hippocampus and it's exciting structure has captivated scientists since its first recorded description by Julius Caesar Aranzio in 1587 who likened it's structure to a seahorse genus *Hippocampus* (Bir *et al.* 2015). It has been a focus of anatomical study throughout the history of anatomy and neuroscience with the father of modern neuroscience, Ramon Y Cajal, spending his time hypothesising its potential functions and then mapping its connections in 1911 (Ramon y Cajal 1911). The hippocampus is found within the medial temporal lobe in mammals. It's curved structure can be found ranging across the dorsal to ventral axis in rodents and from a posterior to anterior axis in humans (Strange *et al.* 2014).

The hippocampus proper has been described as being made up of four regions which are interconnected by the main circuit in the hippocampus, the trisynaptic loop (Figure 1.1). These are the dentate gyrus (DG), cornu ammonis (CA) (further broken down into CA1, CA2 and CA3) and the subiculum. The DG is a C-shaped region with three layers: the granule cell layer (GCL), the molecular layer (ML) and the polymorphic cell layer more commonly referred to as the hilus (Knierim 2015). The key cells of the hippocampus are the glutamatergic granule cells, found in the GCL, whose dendrites reach into the ML to receive input from the entorhinal cortex (EC) through the perforant pathway (which also acts on CA3 directly). The axons of the granule cells extend past the hilus and act on pyramidal cells in CA3 through mossy fibres. CA3 pyramidal cells extend their axons to act on CA1 pyramidal cells through Schaffer collaterals. The CA3 region projects neurons to the EC completing the loop,

as well as the EC neurons projecting back to CA1 and CA3. CA1 is the last stop on the trisynaptic loop and it is the largest source of outputs from the hippocampus with numerous neurons projecting back to the EC and the subiculum (Figure 1.1; Szabo *et al.* 2017). While the DG is primarily comprised of granule cells, there is also a presence of interneurons (which are mostly GABAergic) throughout the hippocampus (Pelkey *et al.* 2017). Primarily the pyramidal basket cell and mossy cells of the DG modulating its signalling and therefore function (English *et al.* 2017).

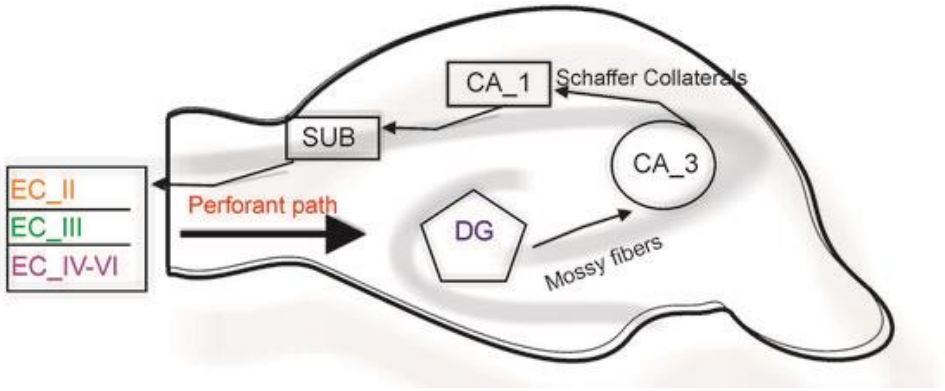


Figure 1.1: Model structure. Information flow in the hippocampus. The perforant path is the major input to the hippocampus. The axons of the perforant path mainly arise in layer II of the entorhinal cortex (ECII). Axons from ECII/IV project to the granule cells of the DG. The mossy fibers are the axons of the DG granule cells and extend from the DG to CA3 pyramidal cells, forming their major input. Information is transferred by axons that project from the CA3 to the CA1 region. The information from CA1 to the subiculum and on the entorhinal cortex (EC) performs the principal output from the hippocampus (Faghihi and Moustafa 2015).

1.1.2 Hippocampus functions

It wasn't until 1957 when a case study was released about patient H.M., who had their hippocampus as well as nearby structures in the medial temporal lobe removed to treat epilepsy, did we first get hints to the potential functions of the hippocampus. Patient H.M., lost their ability to form new declarative memories (Scoville and Milner 1957). This gave scientists a guidepost to begin their investigations into the hippocampus and its function. This insight guided studies into the hippocampus's memory related functions and led to a massive increase in circuit tracing and lesion-based hippocampal studies (Squire 2009). Through these studies we began to learn that the hippocampus is involved in a wide range of functions beyond memory and connections to structures of both affective and cognitive function were soon identified (Squire 2009).

While we first learned that the hippocampus had functions involved with memories and learning novel information (Scoville and Milner 1957), we now know that it has an important role in the initial encoding and later consolidation of new memories in the frontal cortex for long-term declarative memory (that which is consciously recalled) formation and retrieval (Rothschild *et al.* 2017).

The hippocampus is also a cognitive structure which is vital for knowing our sense of where we are. In 2014 Prof. John O'Keefe, Prof. May-Britt Moser and Prof. Edvard I. Moser received the Nobel prize for their work on understanding how the hippocampus fulfils its function in spatial navigation (Burgess 2014). Together they discovered place cells and grid cells which together form the circuitry by which we know our position in relation to our environment. These cells will fire in response to

the exact location they are in in their environment, forming a neuronal circuitry based internal map of our place in our surroundings (Moser *et al.* 2015).

The hippocampus is also heavily associated with emotional functions. It is well established that the hippocampus has connections to the amygdala and the prefrontal cortex. Two regions well known to be involved in emotional computation (Salzman and Fusi 2010). The amygdala is a structure which is involved in contextual fear memory. It is responsible for associating an emotion to a memory (Cohen *et al.* 2013). The hippocampus has been shown to strengthen this process from forming these associations and recalling them (Fastenrath *et al.* 2014).

The hippocampus plays an important role in response to stress. When the body reacts to a stressor, the hypothalamus will activate the sympathetic nervous system to initiate the release of adrenaline from the adrenal glands to facilitate the fight or flight response. Following this process the HPA-axis will activate. It begins with an immediate release of corticotropin releasing hormone and vasopressin from the hypothalamus. These hormones will act on the pituitary gland which will trigger the release of adrenocorticotropic hormone into the bloodstream. This travels from the brain to the adrenal cortex in the abdomen to initiate the release of glucocorticoid stress hormones (cortisol in humans, corticosterone in rats) into the body (Tsigos and Chrousos 2002).

These glucocorticoids act at mineralocorticoid and glucocorticoid (GR) receptors with a high affinity for MR and a low affinity for GR (Nguyen *et al.* 2017). Once the concentration of the glucocorticoid hormone is high enough it will have an action on

GRs. Activation of GRs along the HPA axis and in the hippocampus exerts negative feedback, reducing further release of glucocorticoids.

The hippocampus is highly reactive to glucocorticoids, particularly granule cells in the ventral DG, so when their GR are activated it begins to slow down the firing to react to the stress (Marcuccilli *et al.* 1996; Floriou-Servou *et al.* 2018). This prevents the excessive release of stress hormones when there are no more stressors present. However, this important sensitivity to glucocorticoid stress hormones means that excessive stress may have detrimental effects on many of the other functions of the hippocampus which depend on these granule cells (Joëls *et al.* 2004; Joëls 2008). It is well documented that stress leads to reduced hippocampal volume in rodents (Lee *et al.* 2009; Kim *et al.* 2015).

The dramatic effects of stress on the hippocampus has connected it to stress related psychiatric disorders. Patients with post-traumatic stress disorder, anxiety and major depressive disorder present with a reduced hippocampal volume which is attenuated with antidepressant use (Sapolsky 2000; Sala *et al.* 2004; Boldrini *et al.* 2009; Cobb *et al.* 2013).

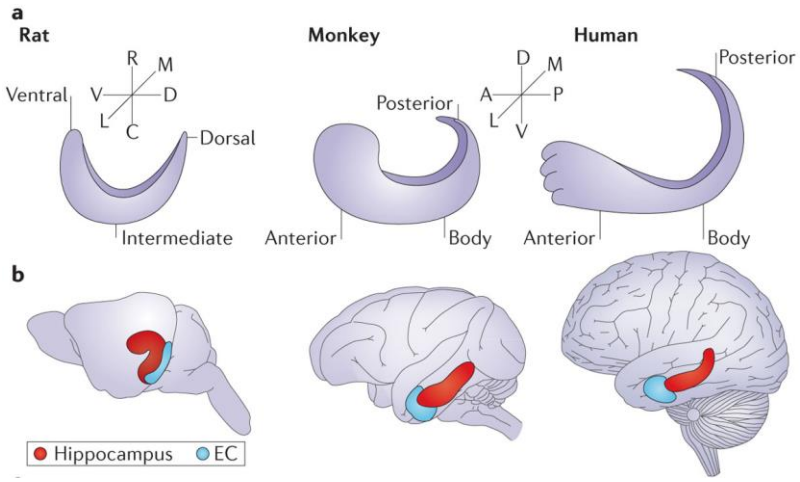


Figure 1.2 (a) Schematic illustrations of the orientation of the hippocampal long axis in rats, macaque monkeys and humans. The longitudinal axis is described as ventrodorsal in rodents and as anteroposterior in primates (also referred to as rostrocaudal in non-human primates). There is currently no precise anatomical definition for a dorsal (or posterior) portion of the hippocampus relative to a ventral (or anterior) one, although in general, topologically, the former is positioned close to the retrosplenial cortex and the latter close to the amygdaloid complex. Note that a 90-degree rotation is required for the rat hippocampus to have the same orientation as that of primates. In primates, the anterior extreme is curved rostromedially to form the uncus. (b) The full long axis of the hippocampus (red) can be seen in brains of rats, macaque monkeys and humans, with the EC shown in blue. A, anterior; C, caudal; D, dorsal; DG, dentate gyrus; L, lateral; M, medial; P, posterior; R, rostral; V, ventral. (Strange *et al.* 2014).

1.1.3 Functional segregation along the longitudinal axis of the hippocampus

Evidence has shown that the hippocampus is functionally segregated along its longitudinal axis into anterior and posterior in primates and dorsal and ventral in rodents (Figure 1.2). This has been demonstrated using hippocampal connectivity studies which show that it connects to many different structures at different connective densities along the longitudinal axis. For example, the dorsal (rodent) /

posterior (primate) hippocampus receives inputs from the anterior cingulate cortex through the EC while the ventral (rodent) /anterior (primate) hippocampus receives most of the connections from the prefrontal cortex, amygdala, and hypothalamus (Strange *et al.* 2014). However, many of these connections are not solely bound to either region, rather their connections to structures are more dominant at certain poles (de Wael *et al.* 2018). For example, connections to the prefrontal cortex occur across the hippocampus. These connections are direct and more numerous in the ventral pole but the dorsal hippocampus also indirectly interacts with the prefrontal cortex (Hoover and Vertes 2007; Parent *et al.* 2009). These observations, as well as genetic expression based studies which suggest further subdivisions of the hippocampus, have led to an understanding where the separate poles of the longitudinal axis of the hippocampus share different connections and therefore are more important for different functions (Christensen *et al.* 2010; Strange *et al.* 2014).

The hippocampus is functionally segregated across the longitudinal axis which has been primarily revealed through lesion-based studies. Lesions of the dorsal hippocampus but not ventral hippocampus impairs spatial memory in rats (Moser *et al.* 1995). Further investigations has found that the hippocampus houses place cells, which are more concentrated in the dorsal two thirds of the hippocampus than the ventral third, that are involved in spatial navigation (Kjelstrup *et al.* 2008). Studies have also shown that the ventral hippocampus is primarily involved in encoding novel stimuli (Greicius *et al.* 2003; DeMaster *et al.* 2013; Collin *et al.* 2015; Dandolo and Schwabe 2018). The ventral hippocampus primarily contributes to emotional functions which are associated with depression and anxiety (O'Leary and Cryan

2014). Bannerman *et al.,* (2003) first found that a cytotoxic lesion to the ventral but not dorsal hippocampus resulted in changes in anxiety behaviours (Bannerman *et al.* 2003). A follow up study found that the contribution of the ventral hippocampus to anxiety behaviours was not dependent on the amygdala and that both structures contribute to regulating anxiolytic behaviour differently (McHugh *et al.* 2004). Rats with ventral hippocampal lesions spend more time in uncovered areas in the elevated plus maze and open field test (Weeden *et al.* 2015). In 1990, Henke *et al.,* (1990) showed that cytotoxic lesions to the ventral but not dorsal hippocampus altered the stress response in rats (Henke 1990). More recently studies have found that stress hormone receptors are more responsive to glucocorticoids in the ventral than dorsal hippocampus, having different effects on synaptic plasticity in each region which may contribute to regulating neural activity and therefore function (Maggio and Segal 2012).

1.2 Hippocampal neurogenesis

1.2.1 Embryonic hippocampal neurogenesis

Early in embryonic development, following the process of gastrulation, the embryo is comprised of three distinct cell layers. The endoderm and mesoderm are the layers from which the abdominal organs, bones and skeletal muscles will arise. The third layer, the ectoderm, gives rise to the skin and the nervous system (Fujinaga *et al.* 1992). On approximately embryonic day 9 in rodents the ectoderm forms the neural tube which marks the beginning of neurogenesis, the process in which neurons are born. The neural tube forms the structure of the early spinal cord and brain and by embryonic day 17.5, almost all of the neurons in the brain have been born (see review by Vijayraghavan and Davidson 2017).

The hippocampus has an interesting developmental sequence as the DG does not share an origin with the rest of the hippocampus. The DG originates in the dentate neuroepithelium which leaves the neural tube and migrates towards the hippocampal neuroepithelium. Between embryonic days 12-15 the hippocampal neuroepithelium engulfs the dentate neuroepithelium and forms the distinct DG raw structure (Altman and Bayer 1990). By embryonic day 17.5 the general anatomical structure of the rodent hippocampus has formed however there is a lot of ongoing development of its neuroarchitecture (Altman and Das 1965; Altman and Bayer 1975). At this timepoint neural precursor cells (NPC) migrate to the hippocampal fissure and form granule cells and NPC cells which will be found in the subgranular zone (SGZ) throughout life (Altman and Bayer 1990).

1.2.2 Postnatal hippocampal neurogenesis

The hippocampus continues to undergo development during the postnatal period. There is evidence that the neuroarchitecture within the hippocampus continues to rapidly develop. For example, synaptogenesis doesn't start until postnatal day (PND) 4 in the rodent DG and finishes in adulthood (Crain *et al.* 1973; Imielski *et al.* 2012). The DG and the GCL are not fully formed and the majority of granule cells do not develop until late in the postnatal period (PND 20-30 in rodents) and insults during this vulnerable time have devastating long-term effects (Altman and Bayer 1990; Piatti *et al.* 2006; Qiu *et al.* 2007; Oomen *et al.* 2009). During the postnatal period, neurogenesis in the hippocampus changes from development of the hippocampal neuroarchitecture into specifically neurogenesis of granule cells in the DG which will persist throughout life. This has been recently identified as a shift in precursor cells (radial glial cells) to form neural stem cells (NSC) and not glia on PND 14 in rodents (Hochgerner *et al.* 2018).

Hippocampal neurons born during late prenatal (embryonic day 19) and early adolescence (PND 21) have been shown to survive into adulthood, however 15% of hippocampal neurons born in the early postnatal period (PND 6) did not survive into adulthood (Ciric *et al.* 2019). The authors have proposed that since these neurons may have a unique life span, they may contribute to hippocampal plasticity. There is evidence that postnatal neurogenesis may contribute to infantile amnesia where these neurons may weaken existing memories and the encoding of information (Josselyn and Frankland 2012; Akers *et al.* 2014). The function of this infantile

amnesia may be to increase the clearance of non-important information and reduce the interference between memories (Kozareva *et al.* 2019).

1.2.3 Adolescent hippocampal neurogenesis

Adolescence is considered to start on PND 21 and end by PND 80 in rodents and start at 11 years of age and end by 25 years of age in humans (Varlinskaya and Spear 2008; Sengupta 2013). There are many different ways in which we define adolescence such as in societal terms where it encompasses the period from the beginning of teenage years until we are legally adults. It can also be defined biologically as the progression through puberty as measured through the Tanner stages (Emmanuel and Bokor 2017) or as the period until the brain is considered fully mature and during which the brain exhibits heightened plasticity (Rubia *et al.* 2000; Shirtcliff *et al.* 2009; Fuhrmann *et al.* 2015). Adolescence can therefore be considered a time before adulthood during which there is ongoing physical, neurological, behavioural and hormonal changes in an individual (Spear 2000).

The number of neurons produced in the hippocampus is very high in adolescence, slows down in adulthood with aging (Kuhn *et al.* 2018). Over 9000 neurons which originate from NSCs in the SGZ are born every day in adolescence while only 700 are born each day in adulthood in humans (Cameron and McKay 2001; Spalding *et al.* 2013). Conversely however, the purpose of an increased level of hippocampal neurogenesis during adolescence is not extensively researched and thus not yet completely understood.

Stress during adolescence and early life is a risk factor for many psychiatric disorders including depression (Costello *et al.* 2003; Schneider 2013). The long term effect of

stress in adolescence on hippocampal neurogenesis is different compared to the effects of stress in adults (see section 1.2.6). These varying effects of stressors on hippocampal neurogenesis across the lifespan, especially in early life and adolescence suggests that researching the long-term effects of stress on hippocampal neurogenesis especially in adolescence is warranted. For example, it has already been shown that fluoxetine, a selective serotonin reuptake inhibitor which is prescribed as an antidepressant, treatment following prenatal stress reverses the development of depressive behaviours and promotes hippocampal neurogenesis in male and female Sprague Dawley rats (Rayen *et al.* 2011).

1.2.4 Adult Hippocampal Neurogenesis

Neurogenesis was originally thought to be a process which was completed solely during gestation and that following birth, humans would not produce new neurons. However, in 1962 Altman found the first evidence that there may be neurogenesis in the postnatal rodent brain (Altman 1962; Altman and Das 1965). He found that there were new neurons being born in the olfactory bulb and the hippocampus of rats. It was another 30 years before such evidence was found in humans. In 1998, Eriksson first detected the presence of newborn cells in the GCL of the hippocampus (Eriksson *et al.* 1998). Research to date has also found that there are potentially up to 700 neurons born every day in the adult human DG capable of integrating into its circuitry (Spalding *et al.* 2013). As already discussed, the adolescent DG produced many more granule cells than the adult DG and it is known that throughout life neurogenesis tends to decrease as rodents age (Spalding *et al.* 2013; Boldrini *et al.* 2018; Kuhn *et al.* 2018; Sorrells *et al.* 2018; Moreno-Jiménez *et al.* 2019).

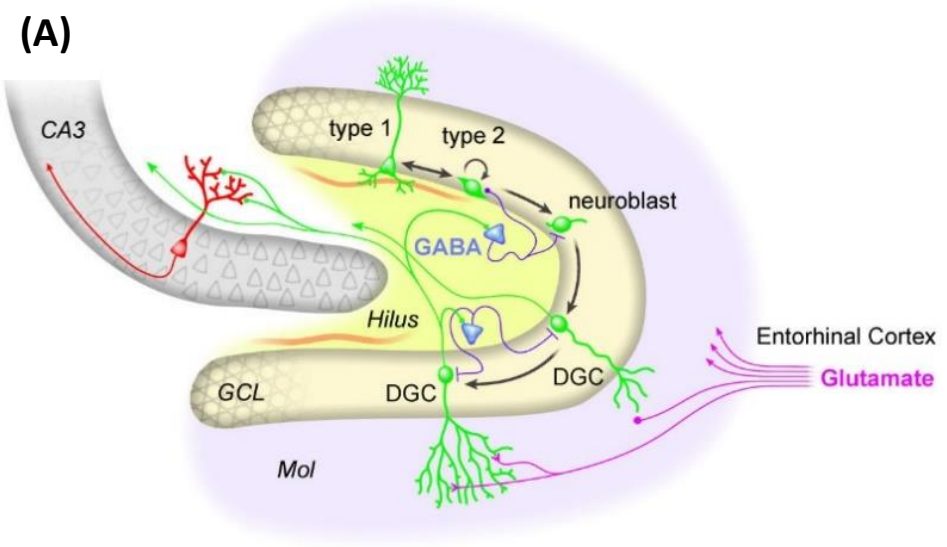
1.2.4.1 How a neuron is born in the adolescent and adult hippocampus

The process by which NSCs in the SGZ of the DG of the hippocampus maintain their population and produce neurons is a very sensitive, complex and sequential process (Figure 1.3A). The first step which is proliferation, refers to the maintenance of NSC population in the DG. This can only happen in the SGZ which is a neurogenic niche. This niche is a very specific environment which provides physiological support to NSCs for their survival and through division to maintain their population (Palmer *et al.* 2000; Bonafina *et al.* 2020). Although the exact distinction between the cells in the SGZ are under debate there is a consensus that there are two types of NSC in the SGZs neurogenic niche. Type 1 cells are similar to radial glial cells and type 2 cells are non-radial (Figure 1.3B; Bonaguidi *et al.* 2012). These NSCs can give rise to NPCs which are limited to proliferation or differentiation into glia or neurons. The main difference between these cells is that NSCs have a far less defined lineage and can proliferate indefinitely but slowly, while NPCs proliferate quickly but are restricted to a certain number of divisions before they differentiate into neurons or glia (Aimone *et al.* 2014).

When NSCs have developed into a NPC and have received signals to undergo differentiation (such as transcription factors NeuroD1, Prox1 and SoxC) they transform into cells called neuroblasts or type 3 cells (Ihunwo *et al.* 2016). These neuroblasts are capable of proliferation but they are now set to a neuronal lineage (Zhao *et al.* 2008). At this point the cells begin to express markers such as doublecortin (DCX). DCX is a microtubule-associated protein which is associated with neuronal migration and is used as a marker for neuroblasts until neuronal maturation which lasts 3 weeks (Saaltink *et al.* 2012). Once the neuroblasts have committed to

neuronal lineage and are now post mitotic, they will begin their migration from the SGZ to the GCL of the DG. These young neurons begin to grow out their axons towards CA3, receive tonic activation from GABAergic interneurons and then inhibitory GABAergic and excitatory glutamatergic inputs as they mature (Zhao *et al.* 2008; Mu and Gage 2011). These maturing neurons extend their dendrites into the ML of the DG, integrate into the circuits of the hippocampus around 2-3 weeks following differentiation and will become functionally indistinguishable from the surrounding neurons after 5 weeks of maturation (Ming and Song 2005; Ming and Song 2011; Mu and Gage 2011).

(A)


CA3
type 1
type 2
neuroblast
GABA
Hilus
GCL
DGC
DGC
Mol
Entorhinal Cortex
Glutamate

(B)

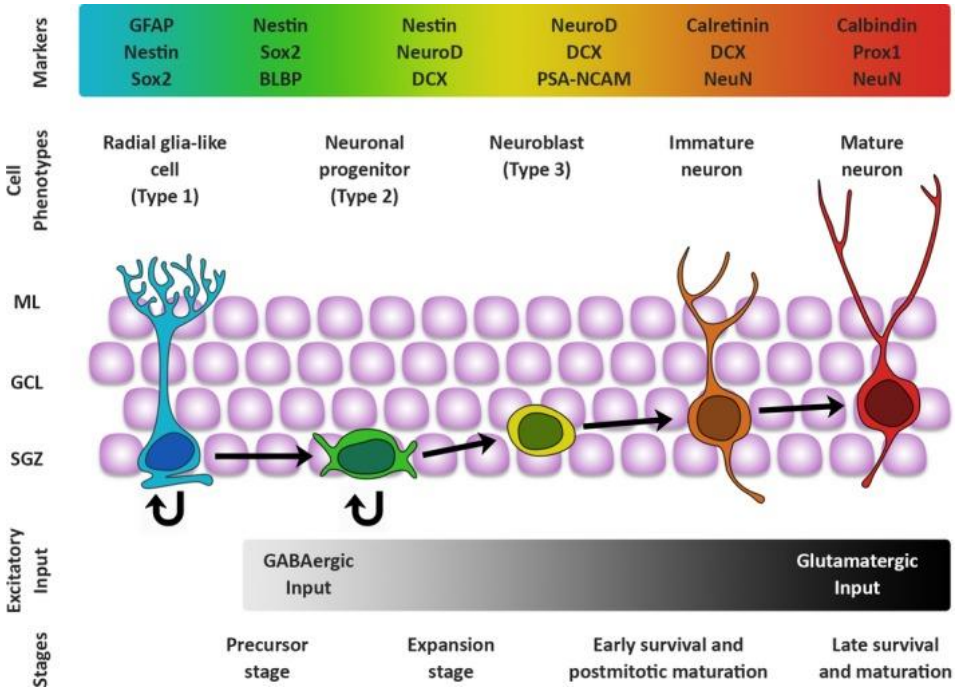

Markers
GFAP Nestin Sox2
Nestin Sox2 BLBP
Nestin NeuroD DCX
NeuroD DCX PSA-NCAM
Calretinin DCX NeuN
Calbindin Prox1 NeuN
Cell Phenotypes
Radial glia-like cell (Type 1)
Neuronal progenitor (Type 2)
Neuroblast (Type 3)
Immature neuron
Mature neuron
ML
GCL
SGZ
Excitatory Input
GABAergic Input
Glutamatergic Input
Stages
Precursor stage
Expansion stage
Early survival and postmitotic maturation
Late survival and maturation

Figure 1.3 (A) Neurogenesis in the adult hippocampus**.** A population of radial cells in the SGZ corresponds to quiescent NSCs (type 1 cells). They coexist with actively proliferating nonradial NPCs (type 2 cells) that generate both astrocytes and neuroblasts. Neuroblasts migrate into the GCL and differentiate into dentate granule cells (DGCs). New-born DGCs gradually develop elaborate dendritic trees in the molecular layer (Mol) to receive inputs from the EC and project to CA3 pyramidal neurons (Habas) as well as hilar interneurons (blue) (Mu and Gage 2011). (B) Stages of hippocampal neurogenesis. Depiction of the stages of the neurogenic process in the hippocampus. The radial glia-like stem cells (Type 1; blue) maintain their pool through self-renewal and give rise to progenitor cells expressing similar markers but displaying different morphology (Type 2 (A&B); green), which undergo rapid proliferation and begin to express markers specific to the neuronal fate of their progeny. Type 2 cells generate neuroblasts (Type 3; yellow). The neuroblasts enter the early survival stage (orange cells) and extend processes towards the molecular layer. During the late survival stage, only newborn neurons that have formed functional connections and have matured morphologically (red cells) remain from the thousands of neuroblasts generated. Granule neuron somata are represented in purple. The colour-coded bar on top illustrates the gradual transition in marker expression as the cells progress through the different stages of the neurogenic process. The grey-gradient-scale bar on the bottom represents the switch of newborn neurons from GABA to glutamatergic input (Kozareva *et al.* 2019). GCL: granule cell layer, ML: molecular layer, NSC: Neural Stem Cell, NPC: Neural Precursor Cell, SGZ: subgranular zone.

1.2.5 A decline in hippocampal neurogenesis with age

The decline of hippocampal neurogenesis from gestation to adolescence and adolescence into adulthood has already been discussed. The trend continues as mammals age (Kuhn *et al.* 1996; Sorrells *et al.* 2018). Studies in rodents have shown that many aspects of neurogenesis begin to decrease with age. Hippocampal proliferation and cell survival, as measured with bromodeoxyuridine (BrdU) injections, a marker for proliferating cells, and neuronal differentiation and maturation, as measured with the markers DCX, a marker for neuronal differentiation and immature neurons, and NeuN, a marker of mature neurons, decreased with age in rodents (van Praag *et al.* 2005). It has also been found that this drop in neurogenesis is met with an increase in gliogenesis (Kuhn *et al.* 1996). It has been theorized that this decline in hippocampal neurogenesis is mostly due to the active reduction of the NSC population with age (Lugert *et al.* 2010). Studies in macaque monkeys have also shown that hippocampal neurogenesis decreases with old age in comparison to rates during adulthood and adolescence (Gould *et al.* 1999). The mechanisms by which aging negatively regulates neurogenesis is reviewed by Kempermann (Kempermann 2015).

The detection of neurogenesis in humans is primarily by immunohistochemical staining of post-mortem tissue. Evidence of neural cellular proliferation is identified by staining for key markers of cell division such as nestin, Ki-67 or Sox-2 (Boldrini *et al., 2018;* Moreno-Jiménez *et al.* 2019; Tobin *et al.* 2019). Whilst evidence of immature hippocampal neurons is primarily identified by staining for DCX or PSA-NCAM and mature neurons, NeuN (Boldrini *et al., 2018;* Moreno-Jiménez *et al.* 2019;

Tobin *et al.* 2019). However, confirmation of the origin of these neurons in rodent and non-human primates is by injection of BrdU, which permanently tags proliferating cells in living organisms. Following sacrifice, staining for immature and mature neuronal markers can be co-localised with stained BrdU to show that DCX-positive, PSA-NCAM-positive or NeuN-positive cells differentiated from a proliferating cell population at the time of BrdU injection (Sorrells *et al.* 2018). Injection of BrdU cannot be administered in human tissue which is a limitation for confirming the origin of immature and mature neurons in the adult human hippocampus. As such, whether hippocampal neurogenesis persists in older age humans has been the topic of much debate and discussion in recent years (Snyder 2018). Many groups appear to produce different results on the issue. Some have failed to find a decline in NSC/NPC cell proliferation or differentiation with aging, and some have failed to find evidence of neurogenesis at all in adulthood (Sorrells *et al.* 2018). However, Boldrini *et al.,* (2018) has shown that neurogenesis persists in adults as old as 79 years of age (Boldrini *et al.* 2018a). Studies by Moreno-Jimenez *et al.,* (2019) and Tobin *et al.,* (2019) have validated these findings in adults showing that adult hippocampal neurogenesis is detectable in human adults aged to the 10th decade of life. Adult hippocampal neurogenesis has also been detected in adult Alzheimer's disease patients aged between 52-97, which was found to be at a lower rate compared to healthy adults (Moreno-Jiménez *et al.* 2019; Tobin *et al.* 2019). This reduced rate of adult hippocampal neurogenesis in Alzheimer's patients was suggested to be associated with Alzheimer's related cognitive deficits which were not present in healthy adults of similar age (Tobin *et al.* 2019).

1.2.6 Functions of postnatal hippocampal neurogenesis

1.2.6.1 The function of hippocampal neurogenesis before adulthood

There have been studies showing that during the juvenile period, the postnatal hippocampus may function to produce the phenomenon of infantile amnesia (Akers *et al.* 2014; Epp *et al.* 2016). These studies have shown that in this early stage of life, the high rate of hippocampal neurogenesis promotes the ability to constantly learn novel information whilst reducing our ability to store memories and appears to be evolutionary beneficial (Josselyn and Frankland 2012). However, there have also been studies with evidence against the neurogenesis hypothesis for infantile amnesia (Barry 2016).

The function of hippocampal neurogenesis in adolescence is not especially well understood. However, a few studies have been conducted directly comparing the impact of various factors including stress on hippocampal neurogenesis in the adult versus adolescent brain (Table 1.1). For example, ablating neurogenesis in females in adolescence can reduce sociability but this effect is not seen when ablating neurogenesis in adulthood (Kirshenbaum *et al.* 2014).

It is important to consider that since adolescence is a time of extreme vulnerability and dramatic physiological change that there are many factors which may be responsible for these time-specific changes. It is of vital importance to continue probing the specific functions of adolescent neurogenesis as it's disruption may hold a key to why trauma in adolescence is such a significant risk factor for the development of stress-related psychiatric disorders (Spear 2000b; Fuhrmann *et al.* 2015; Crews *et al.* 2016).

Table 1.1: Examples of the different immediate effects of extrinsic influences on neurogenesis and rodent response in adolescence vs adulthood. M, Male; F, Female.

Extrinsic influence	Species and sex	Effect in adolescence	Effect in Adulthood	Reference
Ablation of neurogenesis by cranial radiation	Rat, M	↑ Apoptosis ↓Release of growth factors	↑Release of pro-inflammatory cytokines	Blomstrand et al. 2014
Chronic social defeat stress	Mouse, M	↓Proliferation	No change in Proliferation	Kirshenbaum et al. 2014
Whole brain neurogenesis ablation	Mouse, F	↓Sociability	No change in sociability	Wei et al. 2011
Fluoxetine treatment	Rat, M	↑Neurogenesis in the ventral Hippocampus	No effect on neurogenesis	Klomp et al. 2014
High fat diet	Mouse, M	↓Relational memory ↓Neurogenesis	No effect on memory or neurogenesis	Boitard et al. 2012

1.2.6.2 The function of hippocampal neurogenesis in adulthood

We have a much greater understanding of the roles of hippocampal neurogenesis in adulthood as this has been more extensively investigated. In adulthood, neurogenesis continues to have a role in learning and memory. Pattern separation appears to be a key cognitive function of adult neurogenesis. Pattern separation refers to how we separately memorize and recall extremely similar memories (Anacker and Hen 2017). New born neurons exhibit a high degree of plasticity which appears to have a benefit over established neurons in encoding novel memories without affecting retrieval (Appleby and Wiskott 2009; Appleby *et al.* 2011; Déry *et al.* 2013). Studies which ablate hippocampal neurogenesis have also found that without neurogenesis rodents struggle to recall different memories in contexts that are too similar i.e. impaired pattern separation (Clelland *et al.* 2009; Tronel *et al.* 2012).

Adult hippocampal neurogenesis appears to also function to assist the prefrontal cortex in cognitive tasks and enhancing cognitive flexibility (McClelland *et al.* 1995). Studies have shown that when we teach a rat where a platform is in relation to local visual cues they will remember it (Morris water maze) but when the platform is moved, hippocampal neurogenesis is required for quickly relearning the new spatial location of the platform, a measure of reversal learning and cognitive flexibility (Epp *et al.* 2016; Garthe *et al.* 2016). This suggests that adult hippocampal neurogenesis promotes flexibility in learning new things. The hippocampus is connected to the amygdala and this connection is key for encoding a fear response to aversive memories (Anacker and Hen 2017). Adult hippocampal neurogenesis has been

shown to be a crucial component of encoding fear to memories so that there is not an overlap in our emotional reaction to similar memories (Denny *et al.* 2012).

Other studies in which hippocampal neurogenesis has been impaired or enhanced have found that increased hippocampal neurogenesis in rodents is correlated with a better performance in tasks such as spatial learning and memory in the Morris water maze, spatial and object recognition tasks and fear conditioning (Saxe *et al.* 2006; Deng *et al.* 2010). On the other hand, studies have also reported that neurogenesis is not necessary for some of these hippocampal-dependent functions (Groves *et al.* 2013).

1.2.6.3 The function of hippocampal neurogenesis in adulthood across the longitudinal axis

Evidence has accumulated that the function of newly-born hippocampal neurons differs depending on the location of the neuron along the dorsal-ventral axis of the rodent hippocampus. When Kheirbek *et al.,* (2013) assessed the function of granule cells across the dorsoventral axis of the mouse hippocampus using optogenetics to modulate granule cell activity, they found that the function of granule cells in the dorsal and ventral hippocampus differed (Kheirbek *et al.* 2013). Granule cells in the dorsal hippocampus were found to contribute to cognitive flexibility in spatial navigation and increasing granule cell activity in the ventral but not dorsal hippocampus suppressed anxiety (Kheirbek *et al.* 2013). Adult hippocampal neurogenesis has previously been shown to contribute to the spatial processing, where it was not required for learning initial spatial information but rather for reversing previously learned spatial information (Garthe *et al.* 2009). The role of

neurogenesis in the dorsal hippocampus therefore is believed to contribute to cognitive flexibility by processing novel information within a known context (Anacker and Hen 2017).

Kheirbek *et al.,* (2013) were the first to show that increasing the activity of granule cells in the ventral but not dorsal hippocampus reduced anxiety in mice (Kheirbek *et al.* 2013). Studies have shown that increasing neurogenesis, by knocking out a pro-apoptotic gene *bax*, in the ventral but not dorsal hippocampus is associated with reduced anxiety and depressive-like behaviours in mice, suggesting that the ventral hippocampal neurogenesis has an important role regulating these behaviours (Hill *et al.* 2015). Neurogenesis in the ventral hippocampus also contributes to the regulation of the stress response and associated stress resilience (Anacker *et al.* 2018). Anacker *et al.,* (2018) found that newly born hippocampal neurons in the ventral hippocampus inhibit granule cells, and this inhibition prevents the development of anxiety following a chronic social defeat paradigm (Anacker *et al.* 2018). In parallel, there is a growing body of evidence which shows that antidepressants promote neurogenesis in the ventral but not dorsal hippocampus and that this may be a means by which they produce their function (O'Leary and Cryan 2014; Hill *et al.* 2015; Planchez *et al.* 2020). Together these studies suggest that the ventral hippocampal neurogenesis may play an important role in the development of stress-related psychiatric disorders.

1.2.7 Regulation of Hippocampal Neurogenesis

Hippocampal neurogenesis requires a stable environment, which is described as the neurogenic niche, to thrive. This niche within the SGZ comprises glia, vascular-borne intermediates and endothelia which can expose NSCs and intermediate progeny to extrinsic factors which can positively and negatively influence neurogenesis (Rao *et al.* 2008; Ihunwo *et al.* 2016).

Hippocampal NSCs have been shown to be in direct contact with vascular epithelia which produce growth factors (BDNF; brain derived neural growth factor, VEGF; vascular endothelial growth factor) which can subsequently promote hippocampal neurogenesis (Palmer *et al.* 2000; Yang *et al.* 2011; Eisinger and Zhao 2017). Hormones and other signalling molecules including cytokines, stress hormones (cortisol and corticosterone), sex hormones (androgens and oestrogens) which are present in the circulatory system can also affect neurogenesis (Yang *et al.* 2011). Glia, microglia and astrocytes also secrete factors such as BDNF which also influence neurogenesis (Eisinger and Zhao 2017).

It has also been reported that many different types/classes of antidepressant treatments that are used in the treatment of depression and some anxiety disorders increase adult hippocampal neurogenesis in rodents (O'Leary, 2014). In parallel, post mortem human studies suggest that antidepressant medications increase hippocampal neurogenesis in depressed individuals (Boldrini, 2009; Boldrini, 2013). Moreover, using mouse models with impaired neurogenesis it has been shown that at least some of the behavioural effects of some antidepressants are prevented thus suggesting that adult hippocampal neurogenesis plays a role in the mechanism of

action of antidepressant drugs (O'Leary and Cryan 2014). It has also been shown using a mouse model that adult hippocampal neurogenesis is required for antidepressant-induced normalisation of the HPA-axis activity (Surget *et al.* 2011), a finding that might have implications for the precise medicating of individuals with depression, as first time treatments failing are often reported in this stress-related disorder (Kim *et al.* 2019). Finally, there is emerging evidence that stress-induced and antidepressant-induced alterations in adult hippocampal neurogenesis may be segregated along the longitudinal axis, altering predominantly the ventral hippocampus in rodents and the anterior hippocampus of humans (O'Leary and Cryan 2014).

Voluntary exercise has also been shown to be a powerful promoter of hippocampal neurogenesis in rodent studies and this effect has been suggested to occur via increasing the concentrations of BDNF and VEGF (Clark *et al.* 2008; Klempin *et al.* 2013; Bolijn and Lucassen 2015; Ryan and Nolan 2016). Exercise has also been shown to prevent age and stress-induced decreases in neurogenesis possibly through its anti-inflammatory and pro-neurogenic affects in rodents (van Praag *et al.* 2005; Mirochnic *et al.* 2009; Ryan and Nolan 2016). Hippocampal neurogenesis can be increased by nutrient rich diets (Zainuddin and Thuret 2012). This involvement of diet also implicates the gut microbiome as having a regulatory effect on neurogenesis. For example, germ free mice (free of the acquired microbiome) appear to have increased neurogenesis (Stangl and Thuret 2009; Ogbonnaya *et al.* 2015).

Sex hormones have also been shown to regulate adult hippocampal neurogenesis. Long-term exposure to androgens appears to increase neurogenesis cell survival (30-

90 days of exposure but not 15-21 days) and circulating oestrogens have concentration dependent positive effects on proliferation in the rodent DG (Mahmoud *et al.* 2016). Similarly, the stress hormones, the glucocorticoids, also regulate adult hippocampal neurogenesis and it is likely that stress and sex hormones interact to affect adult hippocampal neurogenesis.

1.2.7.1 Hippocampal Neurogenesis and its regulation by the stress response

Rodent NPCs are very sensitive to corticosteroids (Todorova *et al.* 2017). Rats that underwent an adrenalectomy to prevent the release of corticosterone, presented with increased neuronal proliferation and decreased differentiation (Wossink *et al.* 2001). Exposure to acute stressors in adulthood (forced swim test, immobilisation or predator scent) has been shown to decrease hippocampal proliferation in most studies on rats, but not all (Tanapat *et al.* 2001; Wossink *et al.* 2001; Thomas *et al.* 2006). Chronic stress in adulthood is a powerful down regulator of DG neurogenesis. Both long-term psychosocial and physical stressors have been shown to decrease hippocampal neurogenesis at proliferative and differentiating stages of neuron development (Heine *et al.* 2004; Schoenfeld and Gould 2012).

1.2.7.2 Early life stress has a sex specific impact on hippocampal neurogenesis

Early life stress is a common risk factor in the development of many psychiatric and cognitive disorders. There are established sex differences in response to early life stress (Tanapat *et al.* 1999; Lajud and Torner 2015). Immediately following MS (a model of early life stress induced depression), infant monkeys and human adolescent females produce higher glucocorticoid levels than males (Bethea *et al.* 2005; Burghy

et al. 2012). Thus females may exhibit greater sensitivity to neuroendocrinal stress (Burghy *et al.* 2012).

Early life stressors like MS have different effects on levels of neurogenesis in male and female rodents (Table 1.2; Lajud and Torner 2015). The concentration of the female sex hormone, oestrogen may play a role as it varies during the first 21 days of life and has been shown to increase hippocampal neuron proliferation in a concentration dependent manner (Döhler and Wuttke 1975; Tanapat *et al.* 1999; Rummel *et al.* 2010; Tzeng *et al.* 2014). Whilst the impact of early life stress on hippocampal neurogenesis varies depending upon when the stress was applied, the timing of the determination of neurogenesis following early life stress has also produced varying results (Loi *et al.* 2014). Studies determining hippocampal neurogenesis in adult rodents following early life stress predominantly observe reduced hippocampal neurogenesis whilst studies of rodents during juvenile, post-pubertal and adolescence (PND 1-35) have revealed sex-dependent effects of early life stress (Table 1.2; Suri *et al.* 2013; Loi *et al.* 2014). Loi reported that post-natal stress may immediately enhance neurogenesis in male rats whilst reducing neurogenesis in female rats. Sex dependent differences continue to be present in adulthood following post-natal stress where males have reduced neurogenesis and female rats hippocampal neurogenesis recovers (Loi *et al.* 2014).

Table 1.2: Immediate effects of early life stress on neurogenesis in male and female rats (Lajud and Torner 2015).

Early life stress procedure	Species and sex	Age at tissue collection	Effect on hippocampal neurogenesis	Reference
Acute exposure to the odor of a predator on PND 6	Rat, M	PND7	Decreased cell proliferation	Tanapat et al. 1998
Prenatally malnourished pups	Rat, M	PND7, PND30	Reduced cell proliferation PND7, but significantly higher on PND30.	King et al. 2004
24h MS on PN3	Rat, M F	PND4, PND21	Sex differences found between control males and females. PND 4: no changes in proliferation. PND 21: (M) decreased neuronal proliferation, no effect on cell survival, increase in cell differentiation. (F) no effect on neuronal proliferation or neuronal survival, reduced neuronal differentiation.	Oomen et al. 2009
MS15 or MS360 from PND 1-21	Rat, M	PND22	MS360 decreased number of neurons and cell density in the DG, compared to MS15.	Oreland et al. 2010
MS360, (PND 1-14), and early weaning at PND 15	Rat, M	PND28	Decreased cell survival and cell differentiation. Reduced cell density in the DG.	Baek et al. 2011
Early life stress procedure	**Species and sex**	**Age at tissue collection**	**Effect on hippocampal neurogenesis**	**Reference**
MS180 from PND 2 – 14	Rat, M	PND15	MS180 decreased cell survival and cell differentiation.	Lajud et al. 2012

			Reduced cell density in the DG.	
MS 180, PND 2 – 14	Rat, F	PND15	Decreased cell survival in the DG.	Lajud et al. 2012b
Early weaning on PND 14 and later isolation	Rat, M	PND35	Decreased cell proliferation in the DG.	Baek et al. 2012
MS180 from PND 2-21 with or without prenatal nicotine exposure	Rat, M&F	PND14	MS180 increased pyramidal neurons in CA1. In the DG, MS180 decreased number of granule neurons.	Wang and Gondré-Lewis 2013
Prolactin, vehicle, or left undisturbed from PND 1 – 14	Rat, M&F	PND15	No differences in cell number or cell density between control males and females. Prolactin decreased cell survival in the DG.	Lajud et al. 2013

A summary of the studies addressing the effects of early life adversity on different parameters of developmental neurogenesis, indicating sex, type of stress exposure, and age of the rats. CA; Cornu Ammonis, M; Male F; Female, PND; Post Natal Day, MS; Maternal separation, DG; Dentate gyrus

1.3 Neuroinflammation

When the body recognises a pathogen or pathogen-like signal the immune system is activated. An innate immune response is nonspecific, and functions to destroy the pathogen and prepares the body for repair. It does this by raising blood supply to the region causing swelling, heat and pain and recruiting further (initially still non-specific) immune cell action, through the inflammatory response.

Inflammation is controlled by cytokines, which are produced within the central nervous system by activated glia such as microglia, and can have pro-inflammatory or anti-inflammatory properties (Berkenbosch *et al.* 1987; Cassidy and O'Keane 2000; Lucassen *et al.* 2010). Evidence now exists to show that inflammation can regulate behaviour. An example of this is sickness behaviours and how illness alters behaviour to promote survival and recovery (Bouwman and Hawley 2010; Rantala *et al.* 2018; Ellis and Del Giudice 2019). Something which has brought this to the forefront of interest in research on depressive disorders is how sickness behaviour is very similar to depressive behaviour (Maes *et al.* 2012). An enhanced understanding of how the immune system regulates this behaviour has helped researchers understand how depressive behaviours develop (Doosti *et al.* 2013), and potentially lead to targets for drug development.

Neuroinflammation is now recognised as a hallmark of neurodegenerative diseases such as Alzheimer's and Parkinson's (Stephenson *et al.* 2018). Stress-related psychiatric disorders such as depression, anxiety and schizophrenia are also associated with neuroimmune involvement (Ray *et al.* 2017; Van Kesteren *et al.* 2017; Medina-Rodriguez *et al.* 2018).

1.3.1 Microglia: The immune cells of the central nervous system

1.3.1.1 Structure and function

Microglia have two structurally different states (Figure 1.4). In their ramified state, they appear to be star like with long extending processes. These processes increase their surface area, they have an increased pathogen receptor density which increases sensitivity, which enhances the cells ability to monitor of the surrounding area in search of pathogenic material. Although this ramified state is sometimes referred to as the resting state, these cells are active in acting as sentinels surveying the environment, releasing signalling molecules and maintaining homeostasis (Sierra *et al.* 2014).

When microglia in their ramified state detect a signal, they react and initially become activated microglia, which includes undergoing a conformational change into an amoeboid shape (Figure 1.4; Ekdahl *et al.* 2009). In this state, depending on the signal, microglia have been described as classically activated or alternatively activated. Classically activated microglia are involved in pro-inflammatory responses and alternatively activated microglia are anti-inflammatory and are involved in maintaining homeostasis. The state which the microglia may polarise to is specific to the signals the microglia is receiving. For example, lipopolysaccharide (LPS) has been shown to classically activate microglia whilst interleukin-4 (IL-4) alternatively activates microglia (Ekdahl *et al.* 2009; Arsenault *et al.* 2014).

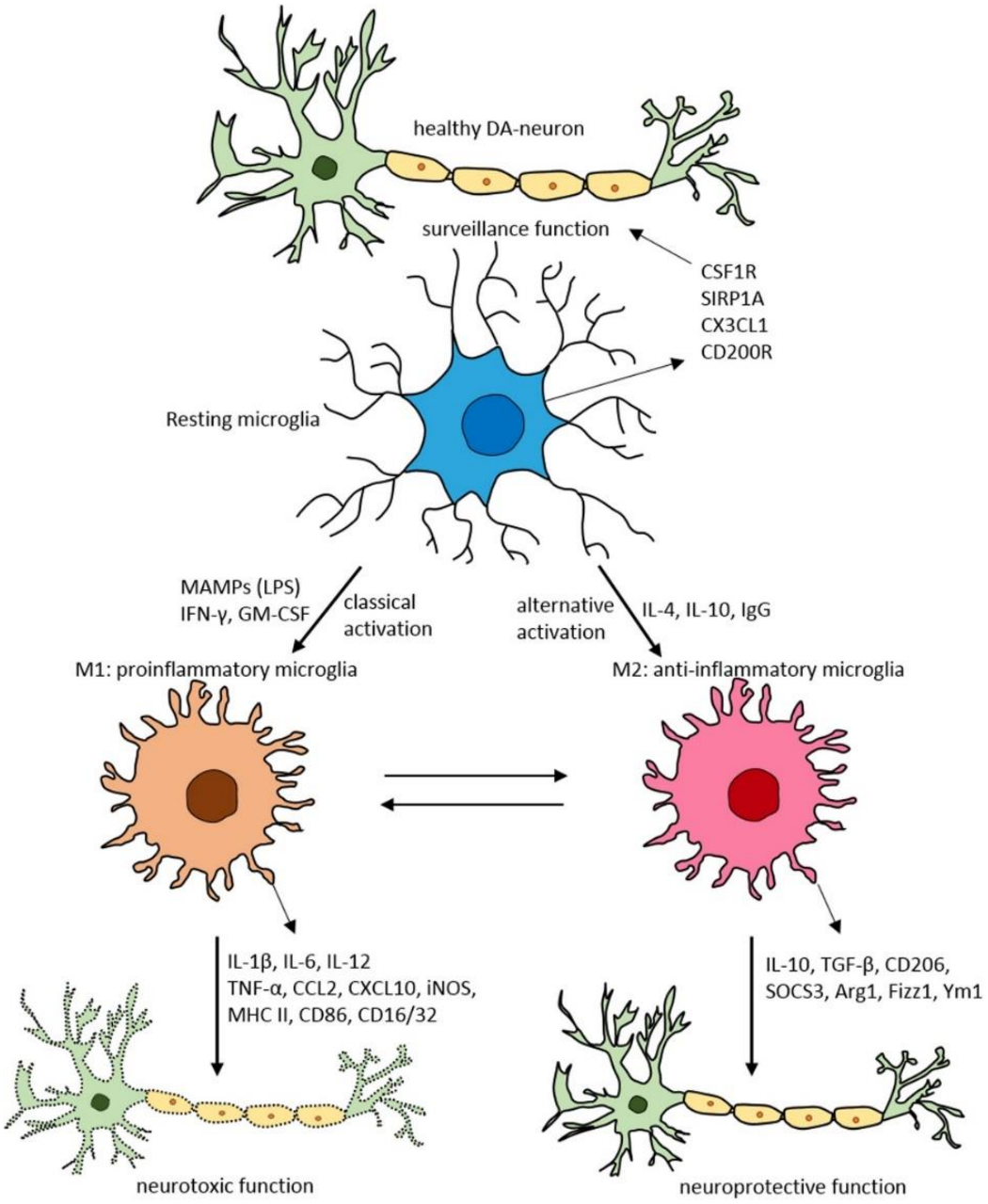


Figure 1.4: Schematic of microglial polarization states and function. In normal physiological conditions microglia acquire the surveillance phenotype to maintain all CNS cell types including neurons. To maintain this surveillance state, microglia secrete several factors including colony stimulating factor 1 receptor (CSF1R), signal regulatory protein CD172 (SIRP1A), chemokine CX3CL1 and CD200R. Upon classical activation when triggered by LPS, IFN-γ, or GM-CSF microglia acquire M1 pro-inflammatory phenotype leading to neurotoxicity by secreting several pro-inflammatory substances. When activated alternatively by IL-1, IgG, or IL-10 microglia acquire an M2 anti-inflammatory state prompting neuroprotection through secretion of variety of

substances (for detailed list, see appendix table 1). Arg1, arginase 1; CCL, chemokine (C-C motif) ligand; CD, cluster of differentiation; CNS, central nervous system; CSF1R, colony stimulating factor 1 receptor; CXCL, chemokine (C-X-C motif) ligand; DA, Dopamine; Fizz1, found in inflammatory zone; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAMPs, microbe-associated molecular patterns; MHC-II, major histocompatibility complex II; SIRP1A, signal regulatory protein CD172; SOCS3, suppressor of cytokine signaling-3; TNF-α, tumor necrosis factor-α; Ym1, chitinase-like protein (adapted from Subramaniam and Federoff 2017).

1.3.1.2 Discovery, Development, Maturation

The developmental origin of microglia has been an area of debate for over a century (Rezaie and Male 2002; Ginhoux *et al.* 2013). W. Ford Robertson first described these central nervous system cells, with a distinct origin from neurons and other known neuroglia (astrocytes and oligodendrocytes), naming them 'mesoglia'. These cells, identified as the 'third element' by Ramon y Cajal, were eventually named microglia by his student Del Rio-Hortega. Hortega eventually further separated and defined microglia from other neuroglia. Recent research has established that the adult microglia population has one distinct origin, the yolk sac (Ginhoux *et al.* 2013).

Within the yolk sac pluripotent cells differentiate into primitive macrophages within blood islands on embryonic day 9 in rodents. The development of the cardiovascular system then allows the colonisation of the brain by primitive macrophages (Ginhoux *et al.* 2013). This yolk sac origin of microglia is conserved across mammals. However, this does not explain the phenomenon of the sudden increase in the population of microglia during the perinatal period (Figure 1.5).

A study recently showed that during the perinatal period (PND 3) fetal liver derived myeloid cells enter the brain and colonize it before undergoing apoptosis in rodents (Prinz *et al.* 2017; Stremmel *et al.* 2018). Another study showed that in the absence of yolk sac derived microglia these fetal liver derived microglia can populate the brain (Ginhoux and Prinz 2015). Bone marrow derived monocytes have been shown to also have the ability to colonise the brain. A study by Beers *et al.,* (2006) showed that when the gene PU.1, necessary for the development of myeloid and lymphoid cells, was knocked out no microglia were present in the brain. However, following a bone marrow transplant on PND 1 these mice developed a fully microglial colonised brain (Beers *et al.* 2006). This further demonstrated the ability and flexibility of peripheral immune cells to pass the blood brain barrier, enter the brain and morph into microglia immediately after birth (Ginhoux and Garel 2018).

During development, from the prenatal stage into adulthood, microglia go through an ontogenic maturation (Bordt *et al.* 2020). Microglia function differs depending on the stage of development. During prenatal development microglia have been shown to promote apoptosis, promote the organisation of neurons and vascularisation in the central nervous system (Paolicelli *et al.* 2011; Pont-Lezica *et al.* 2011). In the perinatal stage microglia have been shown to regulate cell death (Nelson *et al.* 2017). Microglia have been showed to produce reactive oxygen species to promote cell death in new-born neurons and to monitor the population of NPCs in the cerebral cortex perinatally (Cunningham, 2013). They have also been shown to support the growth of cortical neurons as they develop towards their distant targets (Cunningham *et al.* 2013). In adulthood, microglia have been shown to regulate the population pools of neural and oligodendrocyte precursor cells. They are involved in

phagocytosing excess myelin and in maintaining the homeostatic environment of neurons (Hagemeyer *et al.* 2017).

It has also been found that microglia colonise the brain differently in males and females in several regions including the amygdala and hippocampus (Nelson and Lenz 2017). In fact they play a key role in the development of sexually dimorphic centres of the brain such as the preoptic area which underlies sexual behaviour (Schwarz *et al.* 2012). The inhibition of these microglia prevent the development of male sexual behaviour in the future (Lenz *et al.* 2013). The female sex hormone estradiol masculinizes the number and morphology of microglia in females (Schulz and Sisk 2016; Nelson and Lenz 2017). Within the hippocampus, male rats have been found with more microglia than females in the CA1, CA2 and DG. The male microglia also appear to have a less mature morphology. These sex differences in microglial development are significant by PND 4. They are the result of testosterone-mediated cell proliferation in males. Females which receive a treatment of estradiol produce a similar microglial phenotype as males (Nelson and Lenz 2017). Therefore, microglial development is responsive to sex hormones.

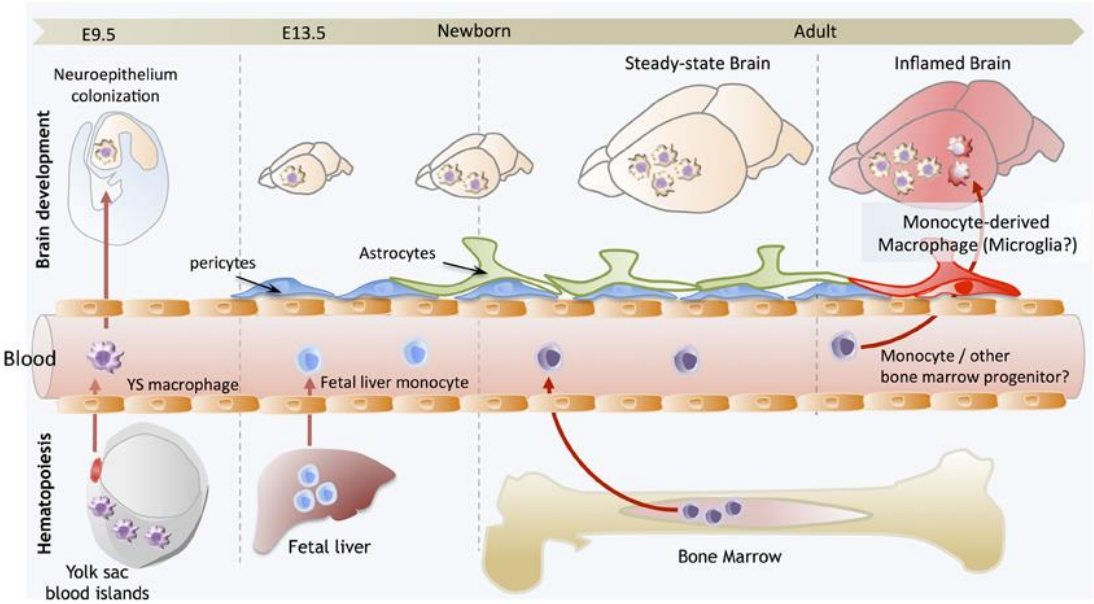


Figure 1.5: Brain development and microglial homeostasis. Primitive macrophages exit the yolk sac blood islands at the onset of circulation and colonize the neuroepithelium from E9.5 to give rise to microglia. The blood brain barrier starts to form from E13.5 and may isolate the developing brain from the contribution of fetal liver hematopoiesis. Embryonic microglia expand and colonize the whole CNS until adulthood. Importantly, in steady state conditions, embryonically-derived microglia will maintain themselves until adulthood, via local proliferation during late gestation and post-natal development as well as in the injured adult brain in reaction to inflammation (adapted from Ginhoux *et al.* 2013).

1.3.2 Regulation of neuroinflammation and microglial activation

1.3.2.1 The regulation of neuroinflammation by stress

Microglia have been shown to be altered in response to glucocorticoids (Frank *et al.* 2012). Glucocorticoids produced by the stress response generally are known to have an immunosuppressive effect (Coutinho and Chapman 2011). Indeed, people who experience occupational stress are more prone to illness and this is hypothesised to be due to this immunosuppressive action of stress hormones (Borritz *et al.* 2006). Glucocorticoids have been shown to promote anti-inflammatory polarisation of

microglia and macrophages (Sugama *et al.* 2013; Xie *et al.* 2019). However, studies demonstrate that the stress can activate microglia to produce pro-inflammatory cytokines (Roque *et al.* 2016; Catale *et al.* 2020). Research has also begun to point towards stress-induced activation of microglia being implicated in the regulation of behaviour and cognition in the both immediate and long term (Walker and Spencer 2018; Horchar and Wohleb 2019).

1.3.2.2 The impact of early life stress on microglia

There is a growing body of research which has identified the potential contribution of the immune system in the development of stress-related psychiatric disorders (Brenhouse and Schwarz 2016; Catale *et al.* 2020). Early life stress has been shown to produce a wide array of effects across age, sex, species and paradigm of stress (Table 1.3). However, early life stress generally leads to an increased pro-inflammatory immunological profile (Delpech *et al.* 2016; Roque *et al.* 2016; Johnson and Kaffman 2018; Desplats *et al.* 2019). It appears that early life stress primes immune responsivity towards a more exaggerated pro inflammatory response to following stressors (Desplats *et al.* 2019).

Michael B. Hennessy has been studying the effects of MS, a model of early life stress induced depression, since 1979 and produced his first paper regarding the potential neuroimmune connection in 2001 (Hennessy *et al.* 2001). He has shown that Guinea pigs which undergo MS produce a characteristic sickness behaviour (which is very similar to depressive behaviours) (Hennessy *et al.* 2007). These guinea pigs were also found to be more sensitized to pro-inflammatory insult later in their life. This behavioural change was showed to be blocked following administration of IL-10 (an

anti-inflammatory cytokine) (Hennessy *et al.* 2007). Since anti-inflammatory cytokine administration rescued the guinea pigs from the development of stress induced sickness behaviours, Hennessy then explored if a pro-inflammatory insult alone was sufficient to produce sickness behaviour and pro-inflammatory sensitization. He found that LPS alone produced sickness behaviour but it did not produce long term sensitisation to inflammatory insult (Perkeybile *et al.* 2009). We can conclude that inflammatory insults alone do not prime long-term effects like early life stressors do.

Early life stress alters the state and number of microglia present in the hippocampus (Table 1.3). MS increases the number of microglia with an activated morphology in the hippocampus of juvenile male rats (Saavedra *et al.* 2017; Banqueri *et al.* 2019). Intriguingly, a study found that this increase in activated microglia as measured by morphological changes did not lead to increased phagocytic activity (Delpech *et al.* 2016). Whilst MS appears to change the morphology of microglia, its effect on the number of microglia is not clear. Studies have found that the number of microglia in the hippocampus, in response to MS, can increase, decrease or remain unchanged (Delpech *et al.* 2016; Roque *et al.* 2016; Saavedra *et al.* 2017; Réus *et al.* 2019). These differences in findings could be due to species, length of separation or the precise age of analysis (Table 1.3). There is evidence that suggests that MS may increase the number of microglia in the brain by reducing cell death rather than increasing proliferation in rats (Chocyk *et al.* 2011). However, social instability stress during adolescence failed to have an effect on the number of microglia in the hippocampus in adolescence or adulthood suggesting that only specific stressors may alter microglial activity (McCormick *et al.* 2012).

Adolescent stress has been shown to have differential immediate and long-term effects with transient increases in microglia activity followed by a decrease in microglial activity markers in adulthood (Catale *et al.* 2020). Brief social isolation of mice on PND 14 increased the number of microglia in the hippocampus however when it was measured in adulthood there was a reduction in the number of microglia in the hippocampus (Gong *et al.* 2018). There is also evidence that stress in adolescence preceded by MS in early life increased the impact of the adolescent stressor on microglia (see review by Catale *et al.* 2020 for more information).

The research into both the immediate and long-term effects of early life stress on microglia is an emerging field which produces varying results dependent on the timing of the stressor and the timing of observation of the microglia (Table 1.3). Furthermore, there is also not enough research into the potential sex differences in the immediate or long-term effects of stress on microglia.

Table 1.3: Effects of postnatal behavioural stressors on hippocampal microglial cell density and phenotype (Catale *et al.* 2020).

Type of stress	Time	Species and sex	Early effects on microglia	Late effects on microglia	Reference
Limited nesting	PND 2-9	Mouse, M	PND 9: ↓ IBA-1+ cells/processes complexity	-	Hoeijmakers *et al.* 2017
Maternal separation	PND 1–21	Mouse, M	P14: ↑ number, activated IBA-1+ cells; PND 14, 28: transcriptomic alterations	-	Delpech *et al.* 2016
	PND 1-14	Rat, M	PND 15: ↓ number, ↑ activated IBA-1+ cells	-	Saavedra *et al.* 2017
	PND 1-10	Rat, M	PND 10: ↑ IBA-1 IR	P20, 30, 60: ↑ IBA-1 IR and mRNA; P40,50: no effect	Réus *et al.* 2019
Social isolation	PND 14-21	Mouse, M F	-	P70: ↓ number, soma size and processes of IBA-1+ cells	Gong *et al.* 2018
	PND 21-63	Rat, M	PND 63: ↑IBA-1 IR, CD11b, ↓ CD200R mRNA	-	Wang *et al.* 2017
Social instability stress	PND 30-45	Rats, M	PND 33,46: no effects on OX-42+ cells number	P75: no effects	McCormick *et al.* 2012

Type of stress	Time	Species and sex	Early effects on microglia	Late effects on microglia	Reference
Maternal separation/restraint stress	PND 1-14, PND 42-56	Mouse, M	PND42: ↑ activated IBA-1+ cells, ↓ CX3CR1 mRNA/PND56: ↑ pro-infl, ↓ anti-infl cytokines mRNA and IR	-	Han *et al.* 2019

A summary of the studies addressing the early and late effects of early life stress and adolescent stress on microglial cell density and phenotype. Abbreviations: Anti-infl: Anti-inflammatory; F: female; IBA-1: ionized calcium-binding adapter molecule; IR: immunoreactivity; M: male; PND: postnatal day; Pro-infl: Pro-inflammtory.

1.3.3 The role of microglia and inflammation in regulating hippocampal neurogenesis

Neuroinflammation and microglial activation is affected by a number of factors which coincidentally also regulate hippocampal neurogenesis, predominantly pro and anti-inflammatory cytokines and glucocorticoids as previously discussed in section 1.3.2. Microglia may have integral functions in the regulation of hippocampal neurogenesis since they are capable of producing both pro- and anti-inflammatory molecules which can decrease and increase hippocampal neurogenesis, respectively (Ekdahl *et al.* 2009; Ryan and Nolan 2016; O'Léime *et al.* 2017). Also, since microglia have been shown to influence neuronal development, as already discussed in section 1.3.1, they may have an even greater role in hippocampal neurogenesis regulation than what is currently known.

1.3.3.1 Regulation of hippocampal neurogenesis by neuroinflammatory insult

Inflammation is a negative regulator of hippocampal neurogenesis. There is a huge amount of evidence that activated microglia have a negative impact on neurogenesis (Ryan and Nolan 2016). Microglia can be experimentally activated using LPS (a chemical which mimics gram-negative bacterial infection) which binds to the toll-like receptor 4 (TLR4) present on immune cells which initiates the release of proinflammatory cytokines like tumour-necrosis factor alpha (Roque *et al.* 2016), IL-1β, IL-6 and other inflammatory molecules (Gayle *et al.* 2004). Interestingly, these individual cytokines have individual effects on stages of neurogenesis; TNF-α (tumour necrosis factor) and IL-1β have been shown to decrease proliferation and differentiation of hippocampal neurogenesis and increase astrocytic differentiation

(Table 1.4; Iosif *et al.* 2006; Green and Nolan 2012), while IL-6 has been shown to decrease proliferation, differentiation and cell survival with no effects on astrocyte development (Kohman and Rhodes 2013). Other IL such as IL-10 increase proliferation and IL-4 increases differentiation (Kohman and Rhodes 2013), whilst resting microglia are involved in the removal of developing dentate granule cells which do not survive into maturity and can release positive neurogenic factors such as insulin growth factor-1 which promotes proliferation (Walton *et al.* 2006; Sierra *et al.* 2010; Kohman and Rhodes 2013). A study recently found that when microglial activation was blocked the negative effect of LPS on adult hippocampal neurogenesis was attenuated (Cai *et al.* 2019).

1.3.2.2 Regulation of hippocampal neurogenesis by inflammation during early life and adolescence

Maternal immune activation produces well established long lasting behavioural outcomes on pups, these pups present with reduced neurogenic potential (Green and Nolan 2014). Decreased hippocampal volume, cell survival, proliferation and differentiation have all been reported in response to pre- and post-natal immune activation (Green and Nolan 2014). LPS administration at PND 5 has damaging long-term effects on neurogenesis such as decreased hippocampal volume, decreased numbers of neurons and an increase in the number of microglia on PND 70 (Wang *et al.* 2013). Administration of LPS on PND 9 showed a decrease in the number of immature neurons and astrocytes in the granular cell layer of the DG and decreased cell survival in the dorsal granular cell layer (Järlestedt *et al.* 2013).

There is limited research on the effects of inflammation on hippocampal neurogenesis in adolescence, but the impact of inflammation during early life on hippocampal neurogenesis at later life stages has been documented (Green and Nolan 2014). Dinel *et al.,* (2014) reported that inflammation in early life, induced by LPS, impairs hippocampal neurogenesis in adolescence and adulthood (Dinel *et al.* 2014). LPS administration to mice at PND 14 has no effect on spatial memory in juvenile and adult mice but increases anxiety like behaviour in adolescence and depressive behaviour in adulthood suggesting that the ventral hippocampus is affected differently to the dorsal hippocampus in response to adolescent inflammation (Dinel *et al.* 2014). Recently, another study found that IL-1β overexpression, beginning PND 28, in the dorsal hippocampus of male rats reduced hippocampal neurogenesis and neurite branching as measured on PND 63 (Pawley *et al.* 2020). However, dorsal hippocampal neurogenesis associated spatial cognition, as measured throughout adolescence by pattern separation (PND 49-58), novel object recognition (PND 59) or spontaneous alternation in the Y maze (PND 60), was not affected by IL-1β overexpression (Pawley *et al.* 2020). Results from the same lab have shown that chronic IL-1β overexpression in the dorsal hippocampus of adult rats impaired pattern separation (Hueston *et al.* 2018). This suggests that dorsal hippocampal neurogenesis related cognition may be resilient to inflammation during adolescence but not adulthood.

1.3.2.3 Sex differences in neuroinflammatory regulation of hippocampal neurogenesis

Prior ideas surrounding why there is a sex difference in neuro-inflammatory outcomes have pointed towards the activity of the female hypothalamic-pituitary-gonadal axis and the female sex hormone estrogen (Loi *et al.* 2014; Bale and

Epperson 2015; Green and McCormick 2016). Estrogen has been shown to have anti-inflammatory effects in the brain (Vegeto *et al.* 2008). Estradiol receptor agonists have been shown to suppress microglial activation following LPS in male and female rats (Arevalo *et al.* 2012). Estradiol also promotes microglial polarisation towards an anti-inflammatory phenotype in the hippocampus of adult female rats (Thakkar *et al.* 2018). Activated pro-inflammatory microglia reduce multiple measures of adult hippocampal neurogenesis, it is therefore possible that the greater activity of estrogen in females may account for sex differences in the regulation of adult hippocampal neurogenesis (Chesnokova *et al.* 2016).

A recent paper by Nelson *et al.,* (2017) found that there are significant sex differences in microglia in the developing hippocampus (Nelson *et al.* 2017). They showed that postnatal hippocampal microglia had a more phagocytic morphology in female rats than males, and that treating females with androgens resulted in microglia with a male-typical morphology (Nelson *et al.* 2017). Following these observations the group explored how this may be impacting adult hippocampal neurogenesis where they found that microglia in females phagocytize more NPCs than male counterparts (Nelson *et al.* 2017). Given that sex hormones fluctuate throughout gestation in rats, such as estradiol surging prior to birth, this could alter microglia to produce these early life sex differences in microglia and therefore hippocampal neurogenesis (Barkley *et al.* 1979).

Table 1.4: Early life hippocampal effects of in utero inflammation (Green and Nolan 2014).

Immunogen	Time of immune challenge	Species and sex	Early life hippocampal alterations	Reference
LPS	E9	Mouse, M F	Increased neuronal density in the CA1 (PND 14)	Nouel et al. 2012
			Increased number of GAD67+ cells in vCA1 of male mice (PND 28)	
Poly(I:C)		Mouse, M F	Decreased number of newly-born neurons in the DG (PND 24)	Meyer et al. 2006
			Decreased number of reelin+ cells in the hippocampus (PND 24)	
		Mouse, M F	Decreased number of reelin+ cells in the dCA1 (PND 28)	Nouel et al. 2012
			Increased number of GAD67+ cells in the vCA1 of female mice (PND 28)	
Poly(I:C)	E9.5	Mouse, M	Decreased axonal size in the CA1 (PND 14)	Makinodan et al. 2008
			Decreased myelin thickness in the CA1 (PND 14)	
LPS	E15	Rat, N/A	Increased number of proliferating cells in the GCL (PND 3-14)	Jiang et al. 2012

Immunogen	Time of immune challenge	Species and sex	Early life hippocampal alterations	Reference

LPS	E15/16	Rat, M	Decreased number of GAD67+ cells in the DG (PND 14 and 28)	Nouel et al. 2012
			Decreased number of reelin+ cells in the DG (PND 14)	
		Rat, M	Decreased cell proliferation in the DG (PND 14)	Cui et al. 2009
			Decrease in cell survival of cells born postnatally (PND 14)	
		Rat, N/A	Increased hippocampal thickness (E18)	Ghiani et al. 2011
Poly(I:C)	E17	Mouse, M F	Decreased number of newly-born neurons in the outer granular layer (PND 24)	Meyer et al. 2006
			Increased apoptosis in the DG (PND 24)	
LPS		Mouse, N/A	Shorter DG (PND 14)	Golan et al. 2005
			Thicker CA1 (PND 7) but thinner CA1 at PND 14	

Immunogen	**Time of immune challenge**	**Species and sex**	**Early life hippocampal alterations**	**Reference**
LPS	E18/19	Rat, M	Decreased DG cell survival born during development (PND 14)	Cui et al. 2009

		Decrease in cell survival of cells born postnatally (PND 14)

A summary of the studies addressing the effect of prenatal immune challenges on the hippocampus in early life. Abbreviations*:* CA – Cornu Ammonis; d – dorsal; DG – dentate gyrus; E – embryonic day; GCL – granule cell layer; LPS – lipopolysaccharide; PND – postnatal day; Poly(I:C) – polyinosinic-polycytidylic acid; v – ventral.

1.4 Aims and Objectives

It has been shown that early life stress alters the expression of both pro and anti-inflammatory cytokine production. Early life stress has also been shown to change the morphology of microglia. However, in the long term it appears to reprogram the immune system towards a more pro inflammatory state in adulthood. Understanding how early life stress alters inflammatory responses may be of great interest for early intervention of stress-related psychiatric disorders with risk in adolescence. The literature also presents a significant gap in our knowledge on the effects of early life stress and inflammation and their interaction on hippocampal neurogenesis and microglial activation, especially in the female brain. Stress-related psychiatric disorders and Alzheimer's disease are more prevalent in females than males, thus it is important to gain further insight into the modulation of hippocampal neurogenesis and microglia by stress and inflammation.

The aim of this thesis is to investigate effects of stress and inflammation during critical periods on hippocampal neurogenesis and hippocampal microglia in male and female rodents. Hippocampal neurogenesis and microglia may play a role in the pathophysiology of depression and anxiety as a result of stressors during critical ontogenetic periods. There is a lot of evidence showing the long-term changes in behaviours in response to early life stress or inflammatory insult but the immediate and long term biological mediators are much less studied. Evidence has also shown the hippocampus as being a key contributor for the sex and age-dependent differences in the prevalence of stress-related psychiatric disorders. The characterisation of hippocampal neurogenesis and co-located microglia following a

stressor during ongoing development may lead to a better understanding of stress-related psychiatric disorders and the development of relevant treatment strategies.

The first objective was to investigate the effects of early life stress and early life inflammation on adult hippocampal neurogenesis and collocated microglia in females in the juvenile period. Specifically, the impact of early life stress induced by MS and inflammation induced by LPS on PND 21 (culled on PND 22) in female rats was investigated. The hypothesis is that MS and LPS would impair hippocampal neurogenesis in the dorsal and ventral hippocampus with a greater degree of impairment in the ventral hippocampus. It was also hypothesised that microglia would attain an activated morphology following MS and LPS, and for LPS to increase the number of microglia in the hippocampus.

The second objective was to investigate the effect of stress during pre-pubertal stages on adult hippocampal neurogenesis and collocated microglia in males and females in adulthood. The impact of a JS paradigm prior to the pubertal window (Horovitz *et al.* 2012) in male and female Sprague-Dawley rats on hippocampal neurogenesis and microglial phenotype in adulthood (PND 83) was investigated. The hypothesis is that hippocampal neurogenesis would not be affected by JS or sex, whilst microglia may have a different phenotype to males due to the influence of sex hormones in the adult rat.

Methods

2.1 Animals

Adult male and female Sprague-Dawley rats (Envigo, UK) were used as breeding partners to generate the sixteen female offspring used in the early life stress and LPS study. After confirmation of pregnancy females were group housed until day 19 after which they were single housed to give birth. In the JS study male and female Sprague-Dawley rats, which were offspring of an ENVIGO breeding stock, were used. Twenty four rats were assigned to non-stressed control or JS groups at random. All animals were housed in the Biological Services Unit, University College Cork housed in a colony maintained at 21 ± 2°C, with a 12:12 hour light-dark cycle (lights on 0630-1830). All animal procedures were performed under authorizations issued by the Health Products Regulatory Authority (HPRA, Ireland), in accordance with the European Communities Council Directive (2010/63/EU) and approved by the Animal Experimentation Ethics Committee of University College Cork.

2.2 Maternal separation

Pups were randomly assigned to the NS or MS groups. This MS procedure which consisted of the separation of the pups from the dams between PND 2-12 starting at 9 am for 180 minutes (Vetulani 2013). The dams were removed first followed by the transfer of the pups to a new cage. The dams were then returned to the original cage. The original cage containing the dams was then moved to another room to prevent olfactory or vocal communication between the pups and dams. Non-stressed rat pups were used as controls to this procedure, each pup underwent up to 5 minutes

of handling to simulate the handling experienced by the rats in the MS group. The NS dams were also briefly removed from their original cage, during which the pups were moved into a new cage and then returned to the original cage. Following the separation procedure animals were left undisturbed. Pups were weaned and sexed at PND 21. Twenty-nine female rats (18 MS + 11 NS) were used in this study. This procedure was carried out by Dr Siobhain O'Mahony (Lecturer and Principal Investigator in the Department of Anatomy and Neuroscience, UCC).

2.3 Lipopolysaccharide administration

On PND 21 half of each of the rats in the MS and NS groups received an intraperitoneal LPS injection (250µg/10mL/kg; Sigma UK) which was made in sterile saline (SAL). The remaining MS rats and NS rats received a volume equivalent intraperitoneal injection of SAL as control at the same time. This resulted in four experimental groups (Table 2.1). The dose and time of assessment after LPS injection was chosen based on literature that shows an elevation of pro-inflammatory cytokine concentrations in the hippocampus (Carvalho *et al.* 2017; Chowdhury *et al.* 2018). This procedure was carried out by Dr Cara Hueston (postdoctoral researcher in Dr Nolan's group. Department of Anatomy and Neuroscience, UCC).

Table 2.1: Experimental groups for the maternal separation and LPS study. NS, Non-separated; MS, Maternal separation; Sal, Saline injected; LPS, Lipopolysaccharide.

Group	n
Control (NS-Sal)	4
Maternal separation (MS-Sal)	4
Lipopolysaccharide (NS-LPS)	4
Maternal separation & Lipopolysaccharide (MS-LPS)	4

2.4 Juvenile stress

Sprague-Dawley rats which were assigned to the JS group were subjected to a 3-day JS paradigm at PND 27-29, as described by Gal Richeter Levins group (Horovitz *et al.* 2012). This begins with a 10 minute forced swim test (circular water tank with of diameter 0.5m; height 0.5m; water depth 0.4m; water temperature 22±2°C) on PND 27. On PND 28 a raised platform stress was performed for 3x30 minute sessions with 60 minutes intervals (the platform is 10cmx10cm at a height of 70cm off the ground). On PND 29 the rats were subjected to a restraint stress for 2 hours (Harvard apparatus 52-0478). Following the completion of each of the above stressors, rats were immediately returned to their home cage. This procedure was carried out by Dr Erin Harris (postdoctoral researcher in Dr O'Leary's group. Department of Anatomy and Neuroscience, UCC). This created four experimental groups (Table 2.2).

Table 2.2: Experimental groups for juvenile stress study. M, Male; F, Female; JS, Juvenile stressed.

Group	n
Male Non-Stressed (M-Non Stressed)	6
Male Stressed (M-JS)	6
Female Non-Stressed (F-Non Stressed)	6
Female Stressed (F-JS)	6

2.5 Tissue collection

For the rats in the MS and LPS study, 24 hours following the LPS or SAL injections, one cohort of rats were euthanized with an intraperitoneal injection of Sleep-Away (1.0 mL/kg) and transcardially perfused with SAL and then 4% (v/v) paraformaldehyde in a 0.1 M phosphate buffer of pH 7.2. The brains were removed and soaked in 4% (v/v) paraformaldehyde at 4°C overnight and then placed into a sucrose solution (30% (w/v)) until sunk, after which brains were frozen at -80°C. The tissue collection was carried out by Dr Cara Hueston (postdoctoral researcher in Dr Nolan's group. Department of Anatomy and Neuroscience, UCC).

The Sprague-Dawley rats which underwent the JS were housed until adulthood. On PND 83 they were sacrificed using sodium pentobarbital (50-90 mg/kg, i.p.). The toe pinch reflex was used to identify when the rats were sufficiently anaesthetised before starting the transcardial perfusion. These rats were first perfused using PBS followed by 4% (v/v) paraformaldehyde to fix the brain tissue. The brains were then removed and post-fixed in 4% (v/v) paraformaldehyde overnight. The brains were then cryoprotected in 30% sucrose before being flash frozen in dry ice and stored at -80 until sectioning. This was carried out by Dr Erin Harris (postdoctoral researcher in Dr O'Leary's group. Department of Anatomy and Neuroscience, UCC).

2.6 Cryostat sectioning

In the MS and LPS study, brains were coronally sectioned (40µm) and mounted onto gelatin coated slides in a 1:6 series through the hippocampus and frozen at -80°C by

Dr Cara Heuston and Ms Tara Foley (Senior Technical Officer in the Department of Anatomy and Neuroscience, UCC).

Brains from the JS study were sectioned into 35µm coronal sections and collected as free floating sections in series of eight and stored in antifreeze (30% ethylene glycol + 25% glycerol in PBS at -20). Sections were then removed from the anti-freeze and underwent 3x30 minute washes in PBS before being transferred onto positively charged slides, dried and then stored at -80 until immunohistochemistry staining.

2.7 Immunohistochemistry

2.7.1 Doublecortin immunohistochemistry

For analysis of newly born neurons sections of hippocampus were stained using an antibody against DCX. Frozen sections were thawed for 10 minutes before rehydration by graded ethanol (100%, 90%, 70% and 50%; 1 minute each). Excess ethanol was removed in a 5-minute deionised water wash. Sections were then blocked for endogenous peroxidases using a 1% hydrogen peroxide (Sigma, 216763) in methanol solution for 40 minutes at room temperature followed by three 10 minute PBS washes. Sections were blocked for non-specific binding using 10% rabbit serum in 0.3% Triton-X in PBS (PBS-T) for 2 hours at room temperature. The sections were incubated in goat polyclonal DCX primary antibody (Santa Cruz; SC-8066) solution (1:100 dilution in 5% rabbit serum in 0.3% PBS-T) at 4°C for 48 hours. Some sections were used as a negative control by incubating with 5% rabbit serum in 0.3% PBS-T without the primary antibody.

Slides were washed in PBS three times for 10 minutes each followed by incubation for 2 hours at room temperature with rabbit anti-goat IgG biotinylated secondary antibody (Vector Labs; PK6105 1:200 dilution) with rabbit serum (3:200 dilution) in PBS-T. Following this the slides were washed in PBS three times for 10 minutes each again before a 2-hour incubation with horseradish peroxidase-streptavidin-ABC complex (Vector labs; PK6105). 3,3'-Diaminobenzidine was (DAB) was activated with 0.03% hydrogen peroxide (Sigma, 216763) which activated the biotinylated complex followed by dehydrations by ethanol series (100%, 90%, 70%; 1 minute each) and a 5-minute histolene wash. Sections were cover slipped with DPX mounting medium.

2.7.2 Ionized calcium-binding adapter molecule 1 staining

To analyse the presence and activation of microglia hippocampal sections were stained for ionized calcium-binding adapter molecule 1 (IBA-1), a microglia/macrophage-specific calcium binding protein. Frozen sections were thawed for 10 minutes before rehydration by graded ethanol (100%, 90%, 70% and 50%; 1 minute each). Excess ethanol was removed in a 5-minute deionised water wash. Sections were then blocked for endogenous peroxidases using a 1% hydrogen peroxide (Sigma, 216763) in methanol solution for 40 minutes at room temperature followed by three 10-minute PBS washes. Sections were blocked for non-specific binding using 3% goat serum in 0.3% PBS-T for 1 hour at 4°C. Sections were incubated in IBA-1 primary antibody (Wako; 190-19741) solution (1:500 dilution of the primary antibody in 1% goat serum in 0.3% PBS-T) at 4°C for 24 hours. Some sections were used as negative controls by incubating with 1% goat serum in 0.3% PBS-T without the primary antibody.

Sections were washed in PBS three times for 10 minutes each followed by incubation for 2 hours with the goat anti rabbit IgG biotinylated secondary antibody (Vector Labs; PK6101) solution with PBS-T. Following this the slides were washed in PBS three times for 10 minutes each again before a 2-hour incubation with horseradish peroxidase-streptavidin-ABC complex at room temperature (Vector labs; PK6101). DAB was activated with 30% hydrogen peroxide (Sigma, 216763) which activated the biotinylated complex followed by dehydrations by ethanol series (100%, 90%, 70%; 1 minute each) and a 5-minute histolene wash. Sections were cover slipped with DPX mounting medium.

2.8 Imaging, cell quantification and cell morphology analysis

2.8.1 Imaging

Images across the hippocampus were visualized at 20x, 40x and 60x using an Olympus BX53 Upright Microscope (Bioscience Imaging Centre, Department of Anatomy and Neuroscience, UCC). The Olympus CellSens software was used to change the focus in order to differentiate between overlapping cell bodies and dendritic crosses. The DG of the hippocampus was separated across the dorsal and ventral axis for analysis. The dorsal DG was defined as between bregma coordinates -1.8 to -5.2mm and the ventral DG between bregma coordinates -5.2 to -6.7mm as previously described (Brummelte and Galea 2010).

2.8.2 Analysis of DCX-positive neurons

The number of DCX-positive cells, their dendritic development and the presence of IBA-1-positive cells in the vicinity of the DCX cell bodies were measured in the DG of the hippocampus (adapted from Nishijima *et al.* 2013). Counting frames were

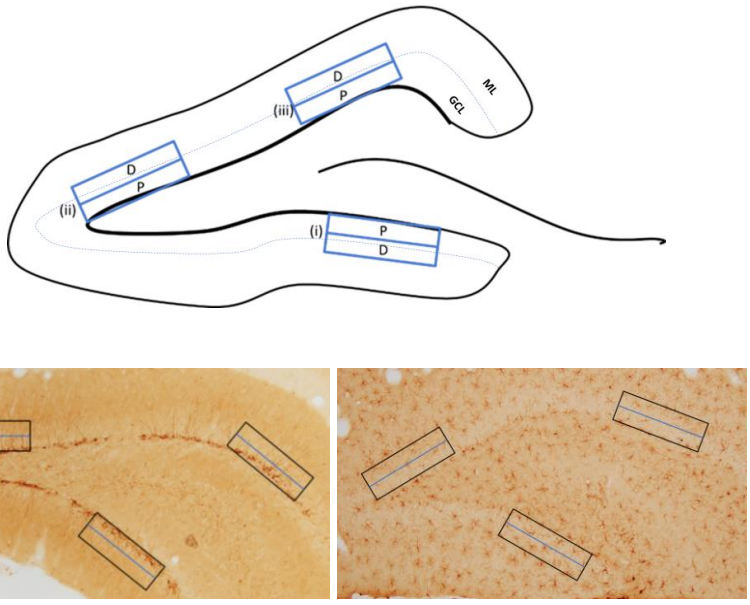
designed such that each pair of distal/proximal frames were aligned; the proximal frame (P) encompassed the GCL and distal frame (D) encompassed the adjacent ML. Each of these frames measured 50µm x 300µm. A cumulative mean analysis was carried out to determine the number of counting frames to be used on the upper and lower blades of the DG for optimal quantification. This resulted in two distal and proximal frames on the upper blade, and one distal and proximal frame on the lower blade of each hemisphere (Figure 2.1A).

For analysis of DCX-positive cells, the number of cell bodies present in the proximal box which was aligned with the GCL was counted. The number of dendrites that crossed from the proximal to distal frame (line 1; proximal dendritic crossing) and the number that emerged out of the distal frame were recorded (line 2; distal dendritic crossing; Figure 2.1B).

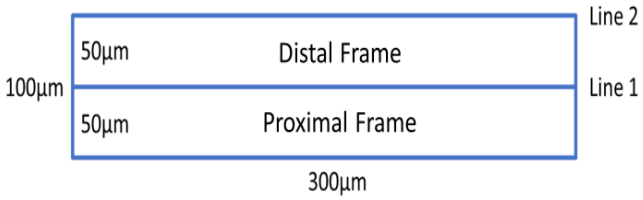
2.8.3 Microglial analysis

The number of microglia present in the proximal and distal frames positioned in the DG was counted (Figure 2.1A). The cell soma size of a randomly chosen IBA-1 positive cell was measured per frame. Six microglia in the dorsal hippocampus and six microglia in the ventral hippocampus per animal were analysed. The area of the soma was then measured using ImageJ and expressed as μm^2.

(A)



(B)



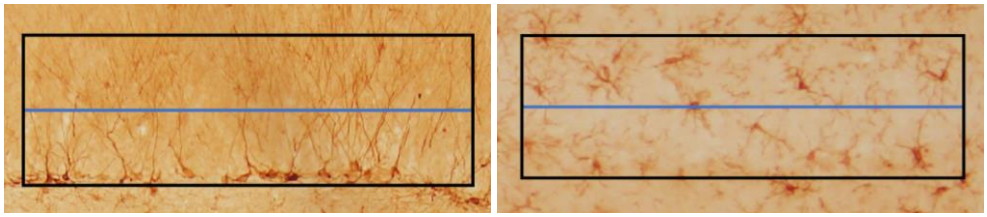


Figure 2.1: (A) Three pairs of frames were overlaid onto each DG image on each hemisphere; one at the lateral end of the lower blade (i), one at the medial tip (ii) and one at the lateral end (iii) of the upper blade. The proximal frame encompassed the granule cell layer and the distal frame encompassed the adjacent molecular layer. (B) The dimensions of each frame. (P; proximal frame D; distal box).

2.9 Data and Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 7.03 for Windows (La Jolla California USA). All datasets were assessed for normal distribution using Shapiro-Wilk test. All data were analysed by a two-way ANOVA. P*ost hoc* Dunnett's multiple comparisons test (LPS administration in MS study) and Fishers LSD multiple comparisons test (JS study) were used to identify where statistically significant differences between the groups were evident. Non-parametric data were analysed with Kruskal–Wallis test followed by *post hoc* Dunn's. An alpha level of 0.05 was used for statistical significance and all data are presented as mean plus standard error of the mean (SEM).

Results

3.1 The effects of maternal separation and LPS on hippocampal neurogenesis in juvenile female rats

To investigate the effects of MS and juvenile inflammation on neurogenesis in the dorsal and ventral hippocampus of juvenile female rats the number and dendritic development of newly born neurons (DCX positive cells) was assessed.

3.1.1 The number of new immature neurons is significantly increased by maternal separation stress but not acute juvenile inflammation in the ventral hippocampus of juvenile females

To assess the effects of MS and juvenile inflammation on neurogenesis in the dorsal and ventral hippocampus of juvenile female rats the number of positively-stained DCX cells was counted.

Two-way ANOVA revealed that there was no effect of MS ($F(1, 12) = 1.201$, $p=0.2947$), LPS ($F(1, 12) = 1.101$, $p=0.3148$) nor a MS x LPS interaction ($F(1, 12) = 0.04403$, $p=0.8373$) on the number of DCX-positive cells across the whole hippocampus (Figure 3.1A). Similarly, upon segregation across the longitudinal axis of the hippocampus there was no effect of MS ($F(1, 12) = 1.337$, $p=0.2701$), LPS ($F(1, 12) = 1.395$, $p=0.2604$) nor a significant interaction between them ($F(1, 12) = 0.01020$, $p=0.9212$) on the number of DCX-positive cells in the dorsal hippocampus (Figure 3.1B). In the ventral hippocampus, there was no effect of LPS ($F(1, 12) = 0.2997$, $p=0.5941$) nor a MS x LPS interaction ($F(1, 12) = 1.433$, $p=0.2544$) on the number of DCX-positive cells in the ventral hippocampus (Figure 3.1C). However,

there was a significant effect of MS ($F(1, 12) = 4.757$, $p=0.0498$) on the number of DCX-positive cells. Subsequent *post hoc* analysis revealed that MS increased the number of newly born neurons in the ventral hippocampus that were treated with SAL (MS/SAL group versus the NS/SAL group ($p<0.05$)) but not LPS.

This analysis shows that the MS, but not LPS, increases the number of DCX-positive cells in the dorsal, but not ventral, hippocampus of juvenile female rats.

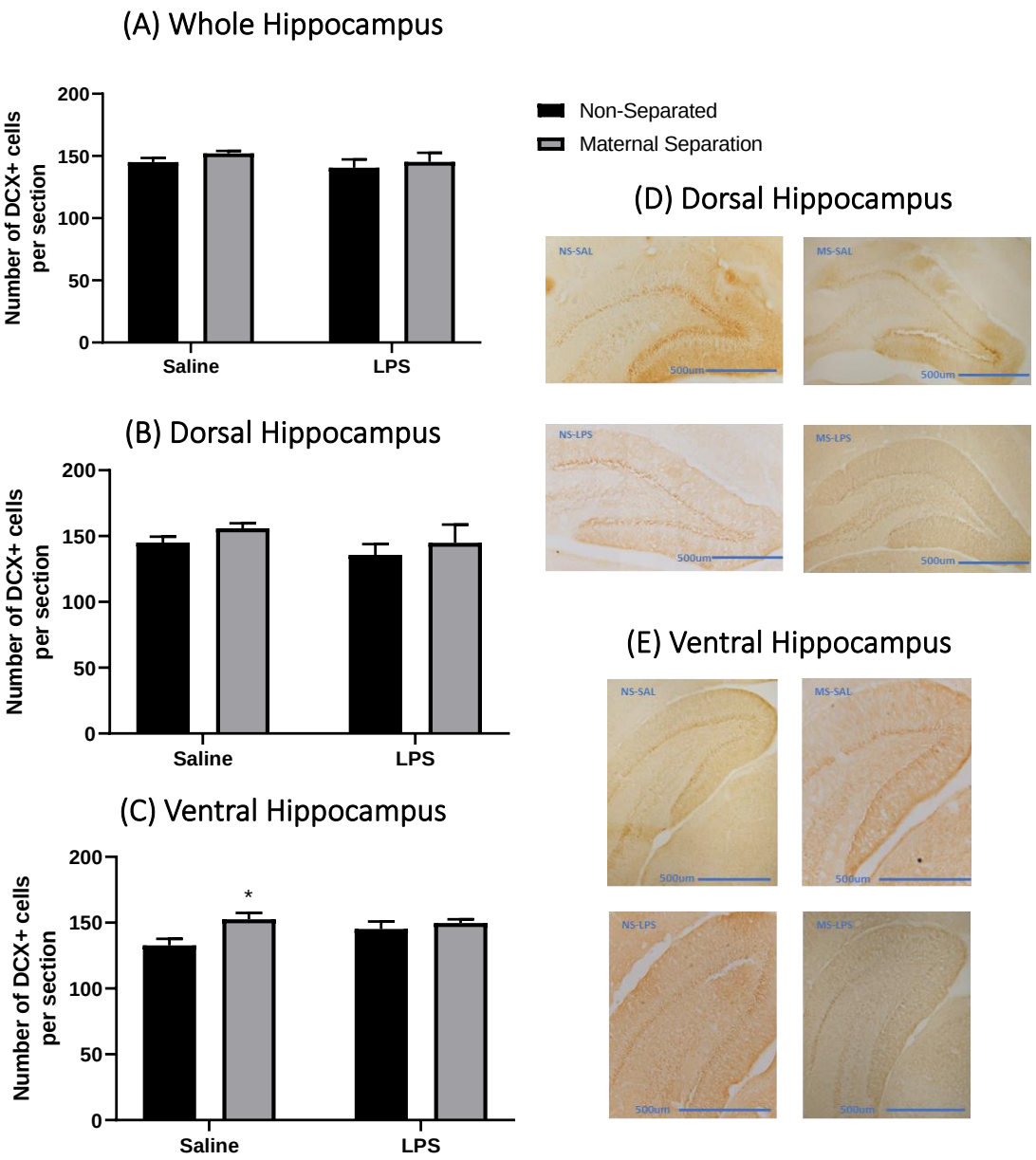


Figure 3.1: Maternal separation increased the number of newly born hippocampal neurons in the ventral but not dorsal hippocampus. There was no effect of maternal separation or LPS on DCX-positive cells in the (A) whole hippocampus or (B) the dorsal hippocampus. Maternal separation significantly increased the number of newly born hippocampal neurons in the (C) ventral hippocampus of saline treated rats. Representative images of the (D) dorsal and (E) ventral hippocampus stained with DCX are shown. Data are shown as mean +/- SEM. n = 4. LPS: lipopolysaccharide; NS: Non-Separated; MS: Maternal separation; Sal: Saline. *vs NS rats of corresponding treatment group, where *p<0.05.

3.1.2 Proximal dendritic crossings in the granule cell layer in juvenile females is significantly reduced by maternal separation and reversed by acute juvenile inflammation

To assess the effects of MS and juvenile inflammation on dendritic development of newly born neurons in the dorsal and ventral hippocampus of juvenile female rats, the number of dendrites of DCX-positive cells which crossed 50µm into the granule cell layer (GCL: proximal dendritic crossing) were counted (Figure 3.2).

Two-way ANOVA revealed that there was no effect of MS ($F(1, 12) = 0.5216$, $p=0.4840$) or LPS ($F(1, 12) = 0.1467$, $p=0.7084$) but there was a significant MS x LPS interaction ($F(1, 12) = 18.63$, $p=0.0010$) on the number of proximal dendritic crossings in the whole hippocampus (Figure 3.2A). Subsequent *post hoc* analysis revealed that MS significantly decreased proximal dendritic crossings in SAL (MS/SAL vs NS/SAL ($p<0.005$)), but not in LPS-treated rats. While LPS decreased proximal dendritic crossings in NS animals (NS/LPS vs NS/SAL ($p<0.05$)), it did not exacerbate MS-induced decreases in proximal dendritic crossings. In fact, LPS reversed the MS-induced decreases in proximal dendritic crossings (MS/LPS vs MS/SAL ($p<0.01$)) and the number of proximal dendritic crossings was also significantly higher in maternally separated/LPS treated animals compared with NS/LPS treated animals (MS/LPS vs NS/LPS ($p<0.05$)).

When the data were segregated across the longitudinal axis of the hippocampus, there was no effect of MS ($F(1, 12) = 0.07885$, $p=0.7836$) or LPS ($F(1, 12) = 0.05873$, $p=0.8126$) on the number of proximal dendritic crossings across the dorsal hippocampus but there was a significant MS x LPS interaction ($F(1, 12) = 8.666$,

p=0.0123) (Figure 3.2B). *Post hoc* analysis revealed that MS significantly decreased the number of proximal dendritic crossings in SAL-treated (MS/SAL vs NS/SAL ($p<0.05$)) but not LPS-treated rats. LPS decreased the number of proximal dendritic crossings in NS rats (NS/LPS vs NS/SAL ($p<0.05$)) but LPS following MS had no exacerbating effect on the number of proximal dendritic crossings across the dorsal hippocampus.

In the ventral hippocampus, two-way ANOVA showed that there was no effect of MS ($F (1, 12) = 1.415$, $p=0.2572$) or LPS ($F (1, 12) = 0.004091$, $p=0.9501$) on the number of proximal dendritic crossings. However, there was a significant MS x LPS interaction ($F (1, 12) = 29.18$, $p=0.0002$) (Figure 3.2C). *Post hoc* analysis revealed that MS decreased the number of proximal dendritic crossings in SAL treated rats (MS/SAL vs NS/SAL ($p<0.005$)) and LPS decreased the number of proximal dendritic crossings in NS rats (NS/LPS vs NS/SAL ($p<0.005$)). However, LPS reversed the MS-induced decrease in proximal dendritic crossings (MS/LPS vs MS/SAL ($p<0.005$)). The number of proximal dendritic crossings was also significantly higher in maternally separated/LPS treated animals compared with NS/LPS treated animals (MS/LPS vs NS/LPS ($p<0.05$)).

This analysis shows that MS or LPS reduces the number of proximal crossings of DCX-positive cells in the hippocampus of juvenile female rats. LPS administration in MS reverses the MS-induced reduction in proximal crossings in the ventral but not dorsal hippocampus.

3.1.3 Distal dendritic crossings in the molecular layer of juvenile females is significantly reduced by maternal separation stress and acute inflammation

To assess the effects of MS and juvenile inflammation on dendrites of new born neurons in the hippocampus of juvenile female rats, the number of dendrites, which crossed 50 μm into the molecular layer (ML: distal dendritic crossings) were counted (Figure 3.2).

Two-way ANOVA showed that there was a significant effect of MS ($F(1, 12) = 10.28$, $p=0.0076$) and LPS ($F(1, 12) = 56.23$, $p<0.0001$) on the number of distal dendritic crossings of new born neurons in the whole hippocampus but MS x LPS interaction did not quite reach statistical significance ($F(1, 12) = 4.583$, $p=0.0535$) (Figure 3.2D). Subsequent *post hoc* analysis revealed that MS decreased the number of distal dendritic crossings in SAL (MS/SAL vs NS/SAL ($p<0.005$)) but not LPS treated animals. LPS significantly decreased the number of distal dendritic crossings in NS (NS/LPS vs NS/SAL ($p<0.0001$)) and in MS animals (MS/LPS vs MS/SAL ($p<0.005$)).

In the dorsal hippocampus, there was a statistically significant effect of MS alone ($F(1, 12) = 23.03$, $p=0.0004$), LPS alone ($F(1, 12) = 57.78$, $p<0.0001$) and a MS x LPS interaction ($F(1, 12) = 29.31$, $p=0.0002$) on the number of distal dendritic crossings (Figure 3.2E). *Post hoc* analysis showed that MS decreased the number of distal dendritic crossings in SAL (MS/SAL vs NS/SAL ($p<0.0001$)) but not in LPS-treated animals. LPS also decreased the number of distal dendritic crossings in NS (NS/LPS vs NS/SAL ($p<0.0001$)) but not maternally separated animals.

In the ventral hippocampus, there was a statistically significant effect of MS ($F(1, 12) = 38.40$, $p<0.0001$), LPS ($F(1, 12) = 35.36$, $p<0.0001$) and MS x LPS interaction ($F(1, 12) = 8.946$, $p=0.0113$) on the number of distal dendritic crossings (Figure 3.2F). *Post hoc* analysis showed that MS decreased the number of distal dendritic crossings in SAL treated animals (MS/SAL vs NS/SAL ($p<0.0001$)). LPS decreased the number of distal dendritic crossings in NS animals (NS/LPS vs NS/SAL ($p<0.0001$)) and it exacerbated the MS-induced decrease in distal dendritic crossings (MS/LPS vs NS/LPS ($p<0.05$)).

This analysis shows that MS, LPS and LPS administration in MS reduces the number of distal crossings of DCX-positive cells in the hippocampus of juvenile female rats. In the ventral hippocampus, LPS administration in MS exacerbates the MS-induced reduction of distal crossings.

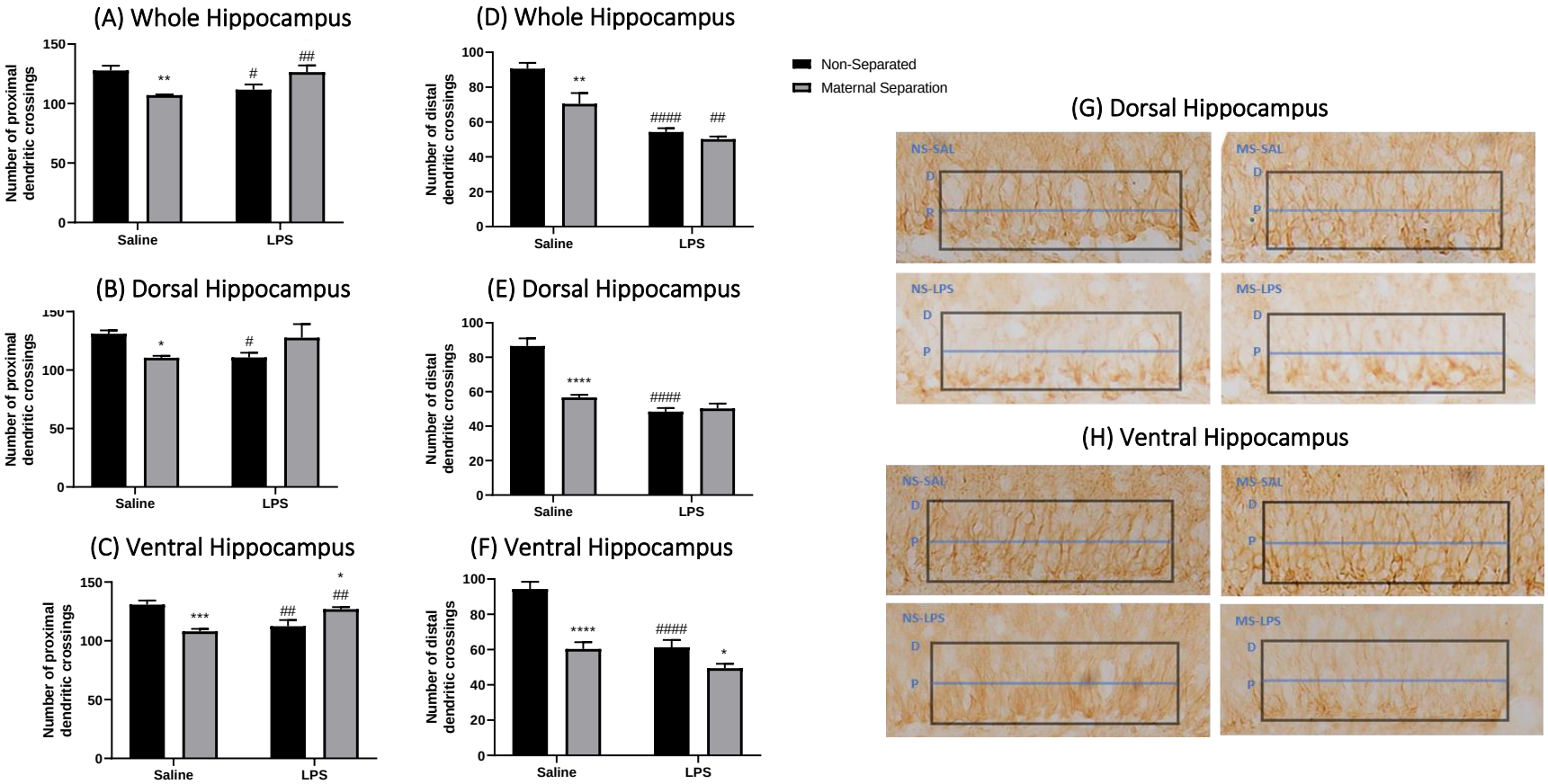


Figure 3.2: The number of proximal dendritic crossings of new born hippocampal neurons into the GCL is reduced by maternal separation or LPS but not by a combination of both in juvenile female rats. Number of proximal dendritic crossings in the (A) whole hippocampus, (B) dorsal hippocampus and (C) ventral hippocampus in juvenile female rats after maternal separation and an acute LPS challenge. The number of distal dendritic crossings into the ML is significantly reduced by stress and inflammation in juvenile female rats in the (D) whole, (E) dorsal and (F) ventral hippocampus. Representative images depicting proximal dendritic crossings in the GCL and distal dendritic crossings in the ML of the (G) dorsal and (H) ventral hippocampus. Data are shown as mean +/- SEM. n= 4. LPS: lipopolysaccharide; MS: Maternal separation; NS: Non-Separated; SAL: Saline; P: Proximal Line; D: Distal line. *vs NS rats of corresponding treatment group, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. #vs saline rats of corresponding treatment group where #p<0.05, ##p<0.01 and ####p<0.0001.

3.2 The effects of maternal separation and LPS on microglia in juvenile female rats

To investigate the effects of MS and juvenile inflammation on microglia in the dorsal and ventral hippocampus of juvenile female rats the number of microglia and their cell soma size were analysed.

3.2.1 The number of microglia is significantly reduced by juvenile LPS following maternal separation in the DG of the dorsal but not ventral hippocampus of juvenile female rats

To assess the effects of MS and juvenile inflammation on microglia number in the dorsal and ventral of the hippocampus of juvenile female rats we counted the number of microglia present in the DG of the hippocampus (Figure 3.3).

A Kruskal-Wallis test revealed that MS, LPS and LPS administration in MS rats significantly effects the number of microglia in the DG of the hippocampus ($H(3) = 8.691$, $p=0.0171$). *Post hoc* analysis found that LPS decreased the number of microglia in the DG following MS (MS/LPS vs NS/LPS ($p<0.05$)) but not in NS animals.

In the dorsal hippocampus a two-way ANOVA showed there was a significant reduction in the number of microglia following MS alone ($F(1, 12) = 5.949$, $p=0.0312$) but there was no effect of LPS alone ($F(1, 12) = 0.3656$, $p=0.5567$) nor a MS x LPS interaction ($F(1, 12) = 2.339$, $p=0.1521$; Figure 3.3B). Subsequent *post hoc* analysis found that LPS decreased the number of microglia in the DG in maternally separated (MS/LPS vs NS/LPS ($p<0.05$)) but not in NS animals.

In the DG of the ventral hippocampus there was no effect of MS alone ($F(1, 12) = 0.6569$, $p=0.4334$), LPS alone ($F(1, 12) = 0.02532$, $p=0.8762$) nor a MS x LPS interaction ($F(1, 12) = 4.401$, $p=0.0578$) on the number of microglia (Figure 3.3C).

This analysis shows LPS administration in MS reduces the number of microglia in the ventral, but not dorsal, hippocampus of juvenile female rats.

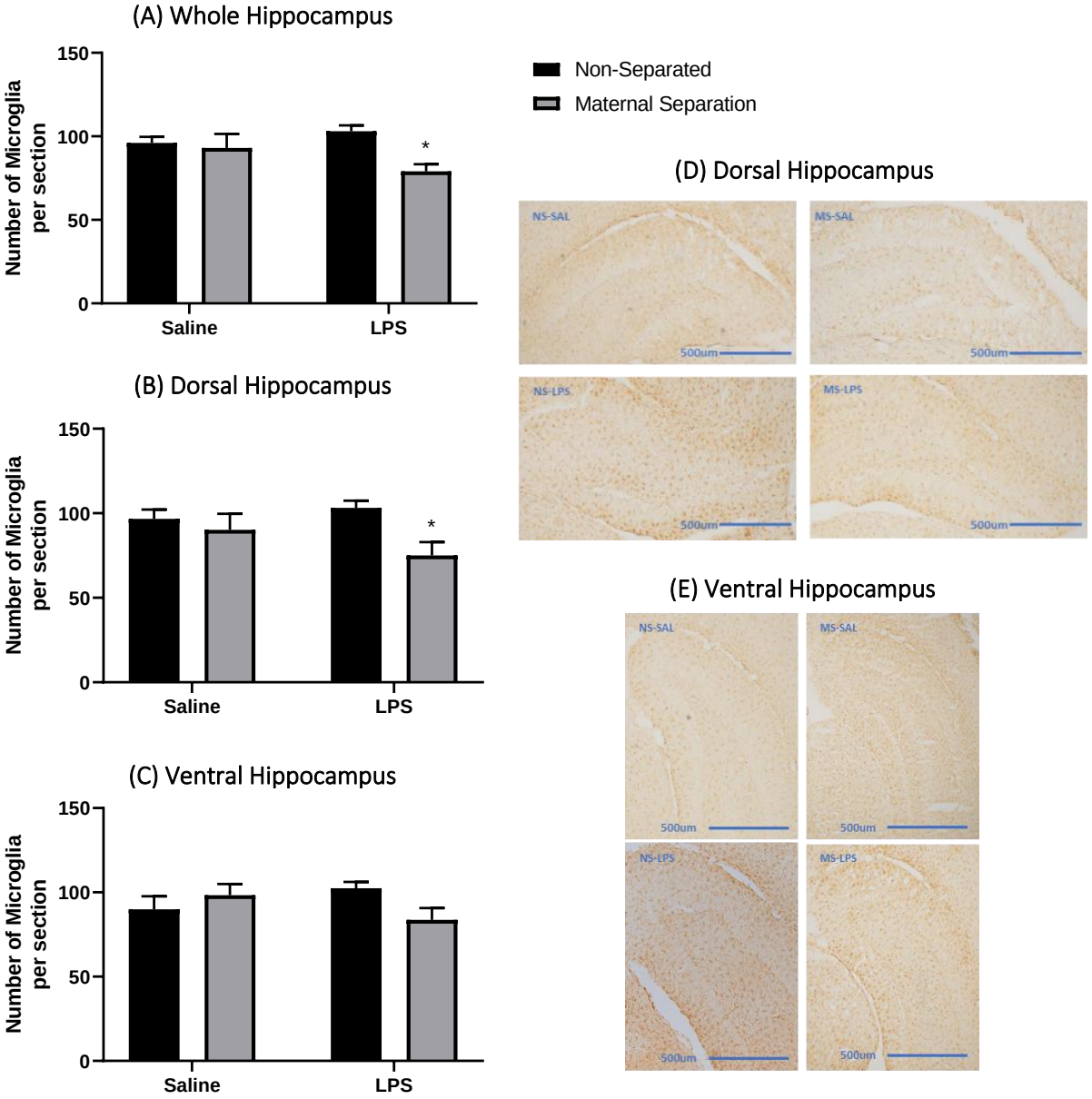


Figure 3.3: Juvenile LPS following maternal separation reduces the number of microglia in the DG of the hippocampus (A) and the dorsal hippocampus (B) but not in the ventral hippocampus (C). Representative images of the (D) dorsal and (E) ventral hippocampus stained with IBA-1. Data are shown as mean +/- SEM. n = 4. LPS: lipopolysaccharide; MS: Maternal separation; NS: Non-Separated; SAL: Saline. *vs NS rats of corresponding treatment group, where *p<0.05.

3.2.2 The number of microglia in the granule cell layer of the hippocampus is not affected by maternal separation or juvenile inflammation in juvenile female rats

To further characterise the effects of MS and juvenile inflammation on microglia number in the dorsal and ventral of the hippocampus of juvenile female rats the number of microglia in the GCL, a subregion within the DG, was assessed (Figure 3.4).

There was no effect of MS ($F(1, 12) = 3.324$, $p=0.0933$), LPS ($F(1, 12) = 0.01491$, $p=0.9048$) nor a MS x LPS interaction ($F(1, 12) = 0.2707$, $p=0.6123$) on the number of microglia in the GCL of the whole hippocampus (Figure 3.4A).

In the dorsal hippocampus there was no statistically significant effect of LPS ($F(1, 12) = 0.0009297$, $p=0.9762$) although the effects of MS ($F(1, 12) = 3.822$, $p=0.0743$), and MS x LPS interaction ($F(1, 12) = 4.076$, $p=0.0664$) showed trends toward statistical significance on the number of microglia in the GCL (Figure 3.4B).

In the ventral hippocampus there was also no significant effect of MS ($F(1, 12) = 0.1887$, $p=0.6717$), LPS ($F(1, 12) = 0.4326$, $p=0.5231$) nor a MS x LPS interaction ($F(1, 12) = 3.513$, $p=0.0895$) on the number of microglia in the GCL (Figure 3.4C).

This analysis shows that MS, LPS or LPS administration in MS has no effect on the number of microglia in the GCL in the hippocampus of juvenile female rats.

3.2.3 The number of microglia in the molecular layer is significantly reduced by juvenile LPS following maternal separation in the dorsal but not ventral hippocampus of juvenile female rats

To further characterise the effects of MS and juvenile inflammation on microglia number in the dorsal and ventral of the hippocampus of juvenile female rats the number of microglia in the ML, a subregion within the DG, was assessed (Figure 3.4).

Two-way ANOVA found that there was no effect of LPS ($F(1, 12) = 0.9505$, $p=0.3488$) but there was a statistically significant effect of MS ($F(1, 12) = 7.921$, $p=0.0156$) and a MS x LPS interaction ($F(1, 12) = 12.98$, $p=0.0036$) on the number of microglia in the ML within the DG of the whole hippocampus (Figure 3.4D). *Post hoc* analysis found that the number of microglia in the ML of the DG was decreased by the double hit of juvenile LPS preceded by MS compared to either MS alone (MS/LPS vs MS/SAL ($p<0.01$)) or LPS alone (MS/LPS vs NS/LPS ($p<0.001$)).

Upon segregation across the longitudinal axis of the hippocampus, LPS had no effect ($F(1, 12) = 0.4566$, $p=0.5120$) whilst MS ($F(1, 12) = 5.445$, $p=0.0378$) and a MS x LPS interaction ($F(1, 12) = 15.45$, $p=0.0020$) had significant effects on the number of microglia present in the ML within the DG of the dorsal hippocampus (Figure 3.4E). *Post hoc* analysis found that LPS increased the number of microglia in the ML of the DG in NS animals (NS/LPS vs NS/SAL ($p<0.05$)). However, LPS decreased the number of microglia in the DG of animals exposed to MS (MS/LPS vs MS/SAL ($p<0.01$)) and MS decreased the number of microglia in the DG of animals injected with LPS (MS/LPS vs LPS/NS ($p<0.001$)).

In the ventral hippocampus, there were no effects of MS ($F(1, 12) = 0.9551$, $p=0.3477$), LPS ($F(1, 12) = 0.08045$, $p=0.7815$) or MS x LPS interaction ($F(1, 12) = 3.721$, $p=0.0777$) on the number of microglia in ML in the DG (Figure 3.4F).

This analysis shows that LPS increases the number of microglia in the ML in the dorsal, but not ventral, hippocampus of juvenile female rats. LPS administration in MS reduced the number of microglia in the ML in the dorsal, but not ventral, hippocampus.

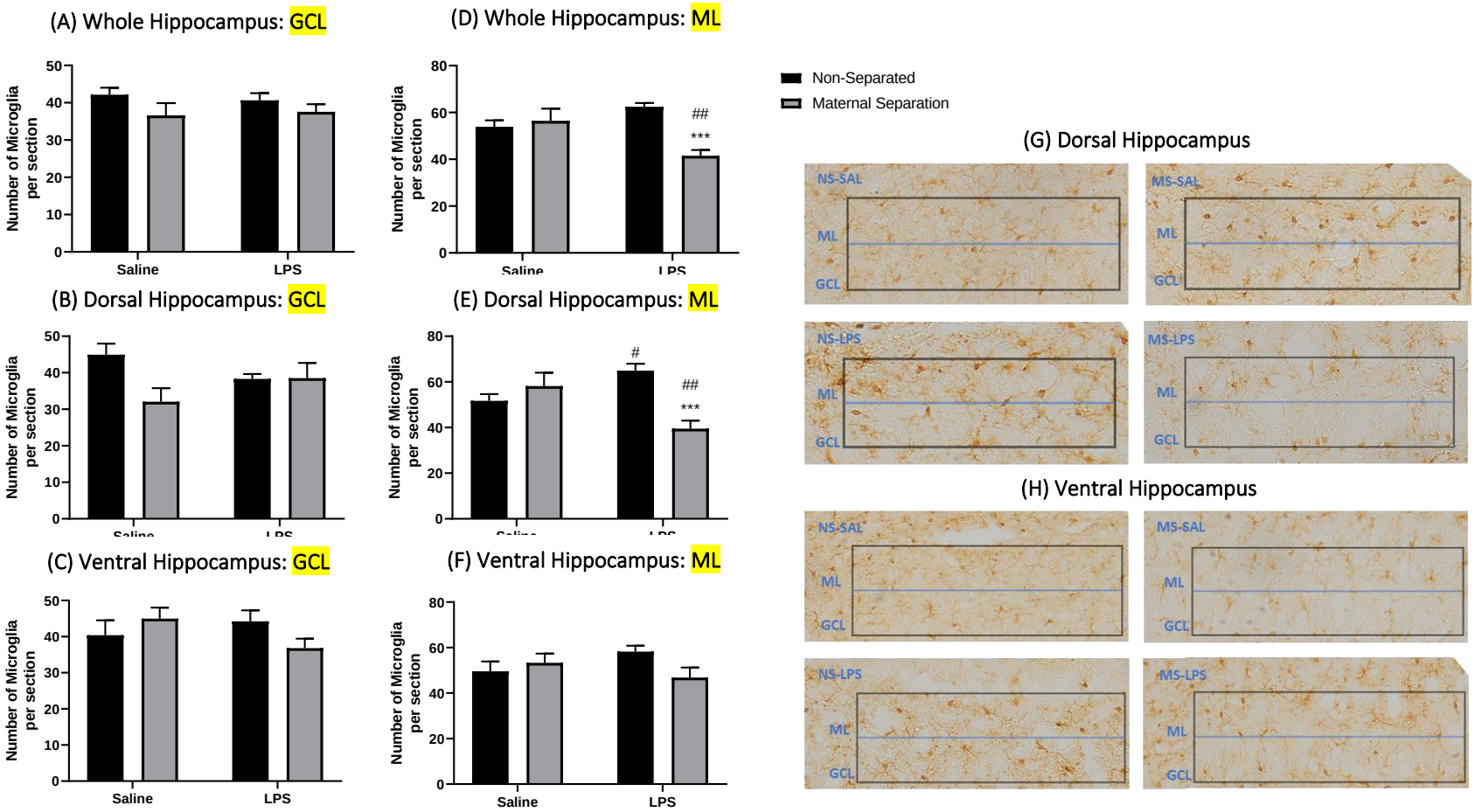


Figure 3.4: The number of microglia in the GCL is not affected by maternal separation or juvenile inflammation across the (A) whole, (B) dorsal or (C) ventral hippocampus. The number of microglia in the ML is significantly reduced by juvenile LPS preceded by maternal separation in juvenile female rats in the (D) whole hippocampus (E) dorsal but not (F) ventral hippocampus. Representative images depicting microglia in the GCL and ML of the (G) dorsal and (H) ventral hippocampus. Data are shown as mean +/- SEM. n = 4. LPS: lipopolysaccharide; MS: Maternal separation; NS: Non-Separated; SAL: Saline; GCL: Granule cell layer; ML: Molecular Layer. *vs NS rats of corresponding treatment group, where ***p<0.001. #vs saline rats of corresponding treatment group where #p<0.05 and ##p<0.01.

3.2.4 The cell soma size of microglia in the DG is increased following maternal separation or juvenile inflammation but the effect of inflammation is attenuated by prior maternal separation

To assess the effects of MS and juvenile inflammation on microglial activation in the dorsal and ventral hippocampus of juvenile female rats we measured the cell soma size of microglia in the DG of the hippocampus (Figure 3.5).

Two-way ANOVA found there was no effect of MS ($F(1, 12) = 0.03092$, $p=0.8633$) on the cell soma size of microglia in the DG of the whole hippocampus. However, there was a significant effect of LPS ($F(1, 12) = 35.15$, $p<0.0001$) and a MS x LPS interaction ($F(1, 12) = 12.48$, $p=0.0041$) on the cell soma size of microglia across the whole hippocampus (Figure 3.5A). Subsequent *post hoc* analysis revealed that MS increased the microglia soma size (MS/SAL vs NS/SAL ($p<0.05$)) in SAL treated animals. LPS also increased microglia soma size (NS/LPS vs NS/SAL ($p<0.0001$)) in NS animals. However, LPS did not increase microglia soma size in maternally separated animals, MS significantly attenuated the increase in cell soma size following LPS (MS/LPS vs NS/LPS ($p<0.05$)).

Upon segregation of the longitudinal axis of the hippocampus, two-way ANOVA revealed that there was no effect of MS ($F(1, 12) = 2.23$, $p=0.1612$) on the cell soma size but there was a significant effect of LPS ($F(1, 12) = 42.26$, $p<0.0001$) and an interaction between MS x LPS ($F(1, 12) = 14.2$, $p=0.0027$) on the cell soma size of microglia in the DG in the dorsal hippocampus (Figure 3.5B). *Post hoc* analysis revealed that MS increased the microglia soma size (MS/SAL vs NS/SAL ($p<0.05$)) in SAL treated animals. LPS also increased microglia soma size (NS/LPS vs NS/SAL

(p<0.0001)) in NS animals. However, LPS did not increase the microglia soma size of maternally separated animals, MS significantly attenuated the increase in cell soma size following LPS (MS/LPS vs NS/LPS (p<0.01)).

In the ventral hippocampus (Figure 3.5C) there was no significant effect of MS (F (1, 12) = 0.079, p=0.7834) whilst again a significant effect of LPS (F (1, 12) = 28.59, p=0.0002) and a MS x LPS interaction (F (1, 12) = 14.23, p=0.0027) was observed. *Post hoc* analysis revealed that MS increased the microglia soma size (MS/SAL vs NS/SAL (p<0.05)) in SAL treated animals. LPS also increased microglia soma size (NS/LPS vs NS/SAL (p<0.0001)) in NS animals. However, LPS did not increase the microglia soma size of maternally separated animals, MS significantly attenuated the increase in cell soma size following LPS (MS/LPS vs LPS/nonseparated (p<0.05)).

This analysis shows that MS and LPS increases the cell soma size of microglia in the hippocampus of juvenile female rats. However, the effect of LPS on the cell soma size of hippocampal microglia was attenuated in MS rats.

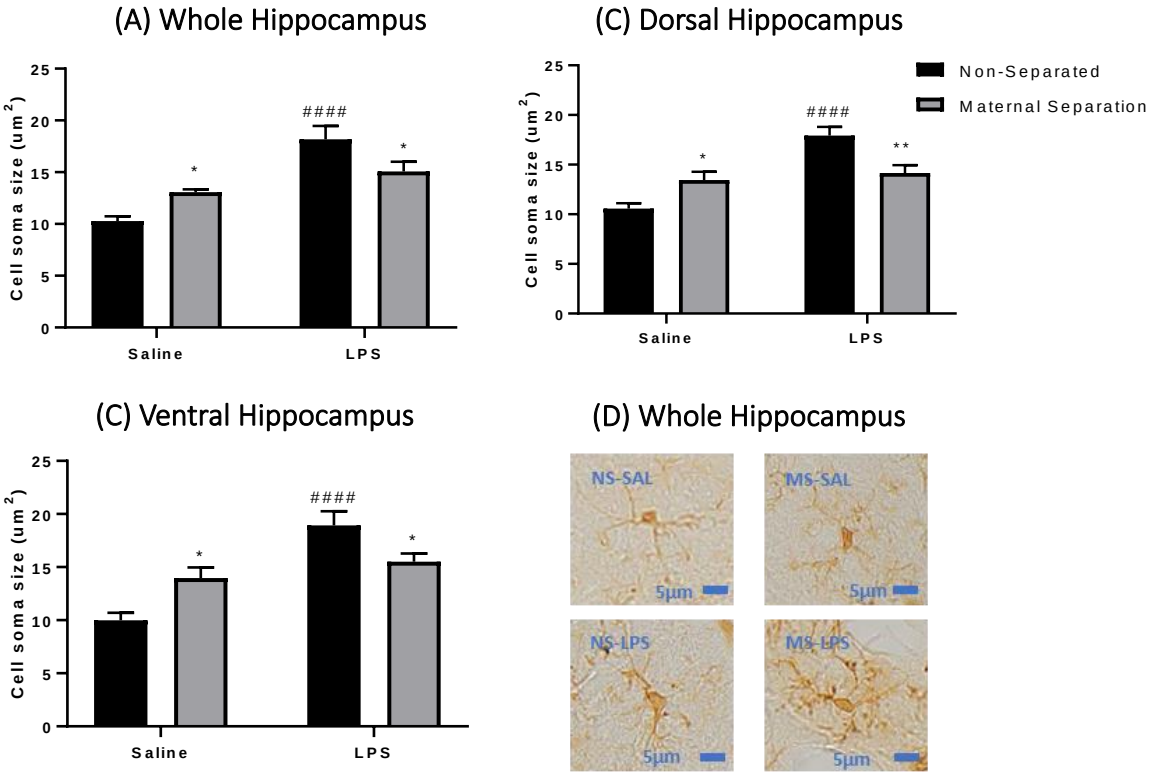


Figure 3.5: Microglia soma size is increased by maternal separation alone and by LPS alone but this effect of LPS is attenuated by prior maternal separation. This is found across the (A) whole, (B) dorsal and (C) ventral DG of the hippocampus. (D) Representative images depicting microglia soma size. Data are shown as mean +/- SEM. n = 4. LPS: lipopolysaccharide; MS: Maternal separation; NS: Non-Separated; SAL: Saline; GCL: Granule cell layer; ML: Molecular Layer. *vs NS rats of corresponding treatment group, where *p<0.05, **p<0.01 and ***p<0.001. #vs saline rats of corresponding treatment group where ###p<0.001.

3.3 The effects of juvenile stress on hippocampal neurogenesis in adult male and female rats

To investigate the effects of JS on neurogenesis in the dorsal and ventral hippocampus of adult male and female rats the number of newly born neurons (DCX-positive cells) and their dendritic development was determined.

3.3.1 The number of new immature hippocampal neurons is lower in females than males but is not affected by juvenile stress in either sex

To assess the effects of JS on hippocampal neurogenesis in adult male and female rats, the number of DCX-positive cells was counted (Figure 3.6). Two-way ANOVA revealed that there was no effect of JS ($F(1, 20) = 0.1764$, $p=0.6789$) nor a stress x biological sex interaction ($F(1, 20) = 0.3017$, $p=0.5889$) on the number of new born neurons in the whole hippocampus. However, there was a significant effect of biological sex whereby females exhibited lower numbers of DCX-positive cells compared to males ($F(1, 20) = 5.412$, $p=0.0306$; Figure 3.6A). However, subsequent *post hoc* analysis did not reveal any significant difference between non-stressed males and non-stressed females ($p=0.2234$) although a trend towards significance was observed between stressed males and stressed females ($p=0.055$).

Upon segregation across the longitudinal axis of the hippocampus, there was no significant effect of JS ($F(1, 20) = 0.3540$, $p=0.5585$) nor a stress x biological sex interaction ($F(1, 20) = 0.6751$, $p=0.4210$) on dorsal hippocampal neurogenesis (Figure 3.6B). Although there was a trend for females to exhibit lower numbers of DCX-positive cells in the dorsal hippocampus compared to males this did not quite

reach statistical significance [Biological Sex: (F (1, 20) = 3.565, p=0.0774)] and thus *post hoc* comparisons were not conducted.

In the ventral hippocampus (Figure 3.6C), there was no effect of stress (F (1, 20) = 0.004560, p=0.9468), nor a stress x biological sex interaction (F (1, 20) = 0.4574, p=0.5066) on hippocampal neurogenesis. Similar to the dorsal hippocampus, neurogenesis in the ventral hippocampus appeared to be lower in females but this did not reach statistical significance (F (1, 20) = 3.524, p=0.0791) and thus *post hoc* comparisons were not conducted.

This analysis shows that adult female rats have reduced DCX-positive cells in the hippocampus than adult male rats.

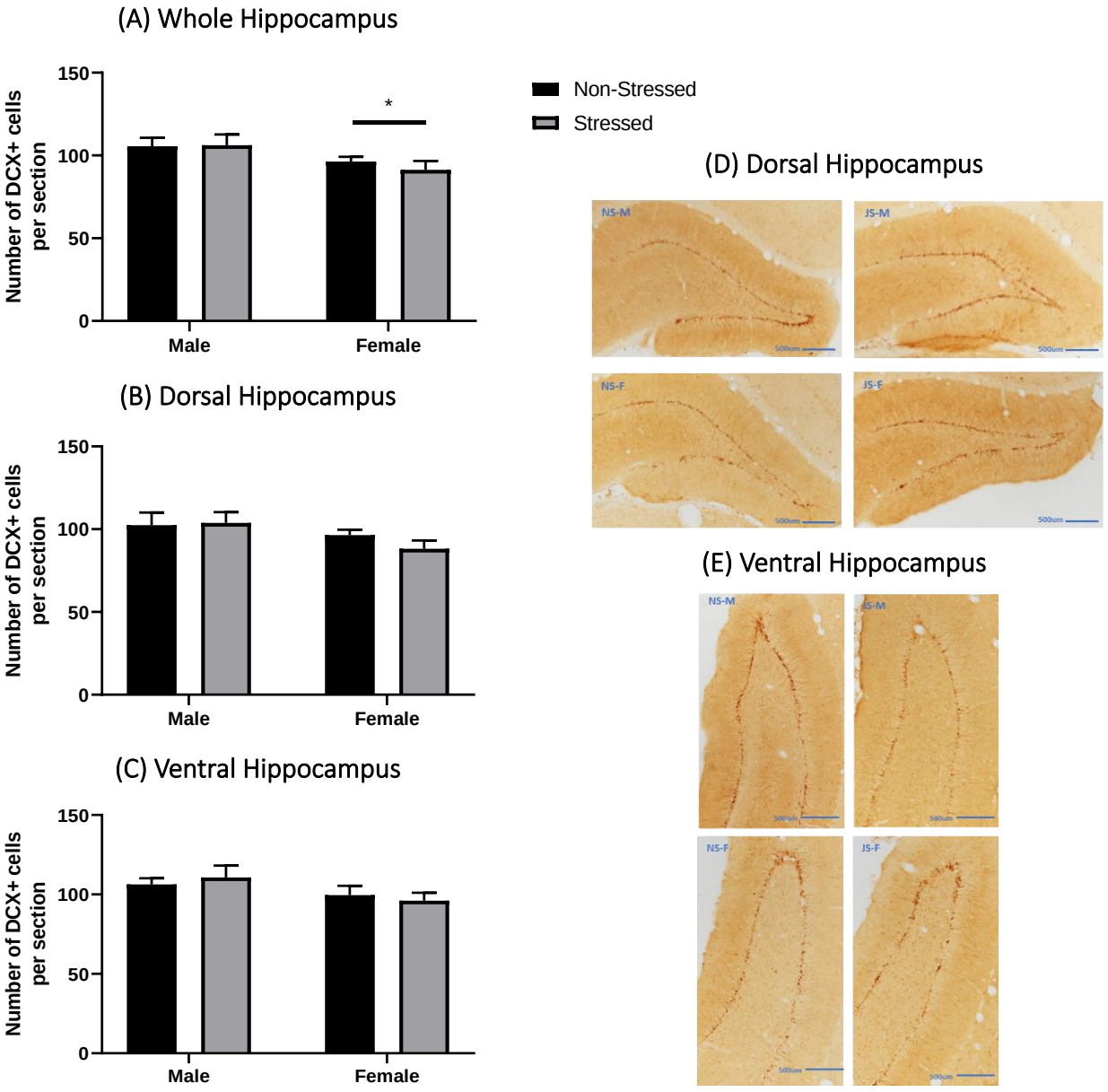


Figure 3.6: The number of new born hippocampal neurons are lower in adult females than males but are not affected by juvenile stress in either sex in the (A) whole, (B) dorsal and (C) ventral hippocampus. Representative images of the (D) dorsal and (E) ventral hippocampus stained with DCX are shown. DCX, Doublecortin; JS: Juvenile stress; NS: Non-Stressed; M: Male; F: Female. Data are shown as mean +/- SEM. n = 6. *Main effect of biological sex, $p<0.05$.

3.3.2 Proximal dendritic crossings of newly born neurons are lower in females but are not affected by juvenile stress either in male or female adult rats.

To assess the effects of JS on dendrites of new born neurons in the hippocampus of adult male and female rats, the number of dendrites of DCX-positive cells which crossed 50 μm into the GCL (proximal dendritic crossings) were counted (Figure 3.7).

Two-way ANOVA revealed that there was no effect of JS ($F(1, 20) = 0.02094$, $p=0.8864$) nor a stress x biological sex interaction ($F(1, 20) = 0.004699$, $p=0.9460$) on proximal dendritic crossings of new born neurons in the whole hippocampus. However, there was a significant effect of biological sex whereby the number of proximal dendritic crossings made by new born neurons was lower in females than in males ($F(1, 20) = 6.203$, $p=0.021$; Figure 3.7A). However, subsequent *post hoc* analysis did not reveal any significant difference between stressed males and stressed females ($p=0.1022$), although a trend towards significance was observed between non-stressed males and non-stressed females ($p=0.0854$).

When the hippocampus was segregated into the dorsal and ventral hippocampus a similar pattern of effects was observed. In the dorsal hippocampus (Figure 3.7B), there was no significant effect of stress ($F(1, 20) = 0.1336$, $p=0.7185$) nor a stress x biological sex interaction ($F(1, 20) = 0.09563$, $p=0.7603$) on proximal dendritic crossings. However, there was a significant effect of biological sex whereby the number of proximal dendritic crossings were lower in females than males ($F(1, 20) = 5.365$, $p=0.0313$). However, subsequent *post hoc* analysis did not reveal any significant difference between non-stressed males and non-stressed females

(p=0.1712) although a trend towards significance was observed between stressed males and stressed females (p=0.0782).

In the ventral hippocampus (Figure 3.7C), there was no effect of stress (F (1, 20) = 0.3897, p=0.5395) nor stress x biological sex interaction (F (1, 20) = 0.04506, p=0.8340) but there was a significant effect of biological sex with lower proximal dendritic crossings in females than males (F (1, 20) = 5.624, p=0.0279). *Post hoc* analysis revealed this effect did not reach statistical significance between non-stressed males and non-stressed females (p=0.0827) or stressed males and stressed females (p=0.1425).

This analysis shows that adult female rats have reduced proximal crossings of DCX-positive cells in the hippocampus than adult male rats.

3.3.3 Distal dendritic crossings of newly born neurons are lower in females but are not affected by juvenile stress in either male or female adult rats.

To assess the effects of JS on dendrites of new born neurons in the hippocampus of adult male and female rats, the number of dendrites which crossed 50 µm into the molecular cell layer (distal dendritic crossings) were counted (Figure 3.7).

Two-way ANOVA showed that there was no significant effect of stress ($F(1, 20) = 0.04475$, $p=0.8346$) nor a stress x biological sex interaction ($F(1, 20) = 0.001535$, $p=0.9691$) on distal dendritic crossings of new born neurons in the whole hippocampus (Figure 3.7D). However, there was an effect of biological sex whereby the number of distal dendritic crossings were significantly lower in females compared to males ($F(1, 20) = 5.937$, $p=0.0243$). However, subsequent *post hoc* analysis did not reveal any significant differences between non-stressed males and non-stressed females ($p=0.1056$) or between stressed males and stressed females ($p=0.0953$).

Upon segregation into the dorsal hippocampus and ventral hippocampus, it was found that in the dorsal hippocampus (Figure 3.7E) there was no significant effect of stress ($F(1, 20) = 0.04475$, $p=0.8346$) nor a stress x biological sex interaction ($F(1, 20) = 0.001535$, $p=0.9691$) on distal dendritic crossings. However, there was a significant effect of biological sex whereby females exhibited lower distal dendritic crossings than males ($F(1, 20) = 5.937$, $p=0.0243$). *Post hoc* analysis did not reveal any significant differences between non-stressed males and non-stressed females ($p=0.2424$) or between stressed males and stressed females ($p=0.083$).

In the ventral hippocampus (Figure 3.7F), there was no significant effect of JS ($F(1, 20) = 0.2728$, $p=0.6072$) nor a stress x biological sex interaction ($F(1, 20) = 0.0005408$,

p=0.9817). There was a significant effect of biological sex whereby new born neurons in females had fewer distal dendritic crossings than males ($F(1, 20) = 5.614$, $p=0.0280$). However, subsequent *post hoc* analysis did not reveal any significant differences between non-stressed males and non-stressed females ($p=0.1062$) or between stressed males and stressed females ($p=0.1127$).

This analysis shows that adult female rats have reduced distal crossings of DCX-positive cells in the hippocampus compared to adult males.

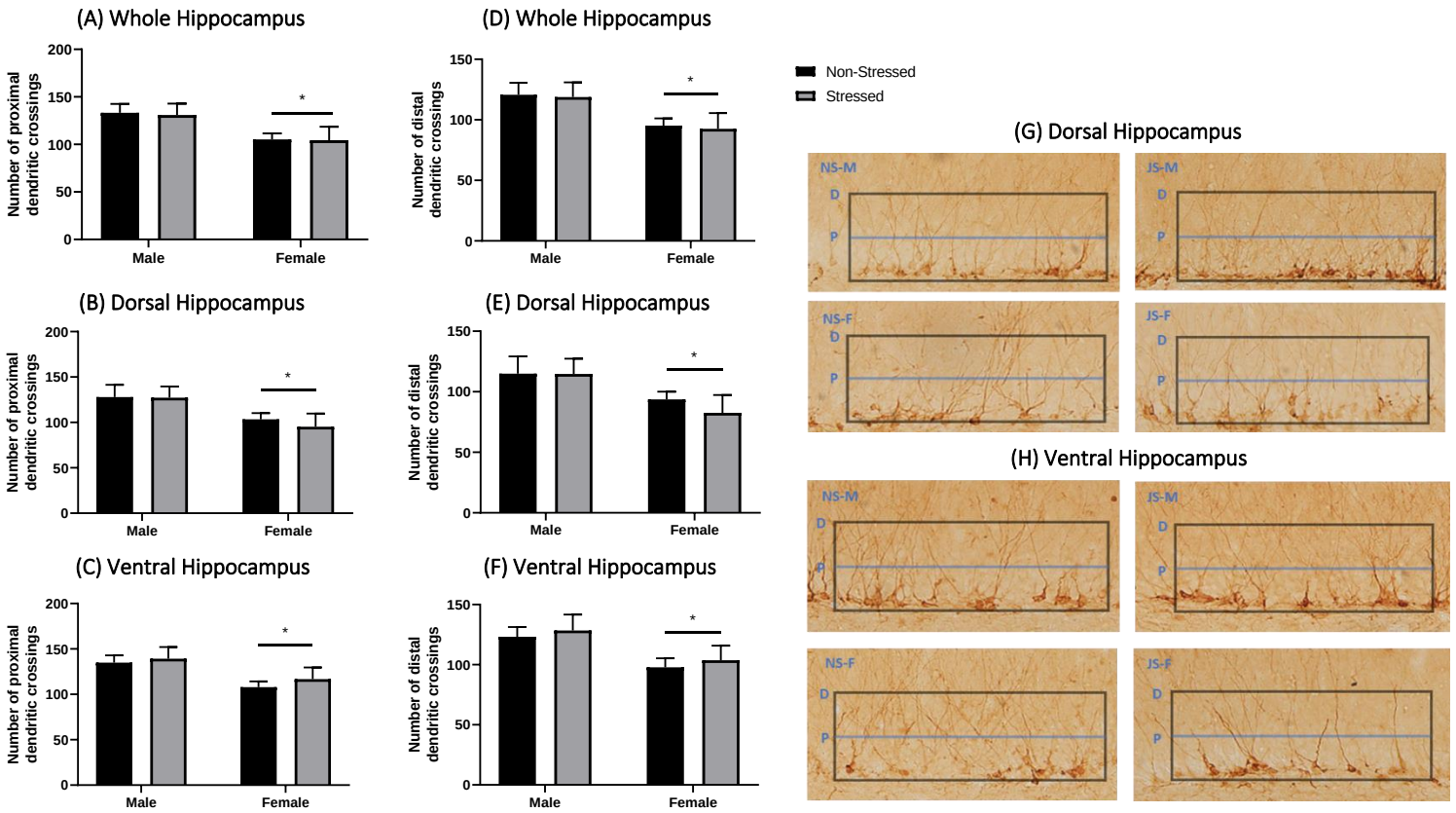


Figure 3.7: The number of proximal dendritic crossings into the GCL from new born hippocampal neurons are lower in females and are not affected by juvenile stress in adult male and female rats across the (A) whole and (B) dorsal hippocampus but not in the (C) ventral hippocampus. The number of distal dendritic crossings into the ML from new born hippocampal neurons are lower in females but are not affected by juvenile stress in either adult male and female rats in the (D) whole, (E) dorsal and (F) ventral hippocampus. Representative images depicting proximal and distal crossings in the (G) dorsal and (H) ventral hippocampus. Data are shown as mean +/- SEM. n = 6. DCX: doublecortin, JS: Juvenile stress; NS: Non-Stressed; M: Male; F: Female; P: Proximal line; D: Distal line. *Main effect of biological sex, $p<0.05$.

3.3.5 The average number of proximal dendritic crossings per newly born neuron are lower in females but are not affected by juvenile stress in either male or female rats.

The data in section 3.3.2 suggests that there were less proximal dendritic crossings in females than males. However, this finding might also be due to the fact that the number of DCX-positive cells was lower in females compared to males. Thus, to account for this potential confounding factor the average number of proximal dendritic crossings per DCX-positive cell was calculated (Figure 3.8). This would give some insight into whether males and females differed in the dendritic complexity of their newly born neurons.

Two-way ANOVA showed no significant effect of stress ($F(1, 20) = 0.006379$, $p=0.9371$) nor a stress x biological sex interaction ($F(1, 20) = 0.2598$, $p=0.6158$) on average number of proximal dendritic crossings per neuron in the whole hippocampus (Figure 3.8A). The number of proximal dendritic crossings per neuron were found to be significantly reduced in females compared to males [Biological Sex ($F(1, 20) = 4.580$, $p=0.0449$)]. Subsequent *post hoc* analysis did not reveal any significant differences between non-stressed males and non-stressed females ($p=0.0757$) or between stressed males and stressed females ($p=0.2626$).

When the hippocampus was segregated into the dorsal and ventral hippocampus, there was no effect of stress ($F(1, 20) = 0.06372$, $p=0.8033$) nor a stress x biological sex interaction ($F(1, 20) = 0.0003522$, $p=0.9852$) on proximal dendritic crossings per neuron in the dorsal hippocampus (Figure 3.8B). However, there was a significant effect of biological sex whereby females exhibited less proximal dendritic crossings

per neuron than males in the dorsal hippocampus ($F(1, 20) = 4.584$, $p=0.0448$) with females presenting with less proximal dendritic crossings per neuron in the dorsal hippocampus compared with males. However, subsequent *post hoc* analysis did not reveal any differences between non-stressed males and non-stressed females ($p=0.1414$) or between stressed males and stressed females ($p=0.149$).

In the ventral hippocampus (Figure 3.8C) there was no significant effect of stress ($F(1, 20) = 0.5466$, $p=0.4683$), nor a stress x biological sex interaction ($F(1, 20) = 1.031$, $p=0.3220$). Although it appeared that the number of number of proximal dendritic crossings per neuron were lower in females compared to males, this effect did not quite reach statistical significance (Biological Sex: $F(1, 20) = 3.583$, $p=0.0730$) and thus *post hoc* analysis could not be conducted.

This analysis shows that adult female rats have reduced proximal crossings per DCX-positive cell in the dorsal, but not ventral, hippocampus compared to adult males.

3.3.5 Distal dendritic crossings per neuron are lower in females but are not affected by juvenile stress in male and female rats.

The data in section 3.3.3 suggests that there were less distal dendritic crossings in females than males. However, this finding might also be due to the fact that the number of DCX-positive cells was lower in females compared to males. Thus, to account for this potential confounding factor the average number of distal dendritic crossings per DCX-positive cell was calculated (Figure 3.8). This would give some insight into whether males and females differed in the dendritic complexity of their newly born neurons.

Two-way ANOVA revealed no effect of JS ($F(1, 20) = 0.2233$, $p=0.6417$) nor a stress x biological sex interaction ($F(1, 20) = 0.0008433$, $p=0.9771$) on the number of distal dendritic crossings per neuron in the whole hippocampus (Figure 3.8D). However, the number of distal dendritic crossings per neuron were significantly lower in females compared to males [Biological sex: $F(1, 20) = 4.854$, $p=0.0395$). However, subsequent *post hoc* analysis did not reveal any differences between non-stressed males and non-stressed females ($p=0.1301$) or between stressed males and stressed females ($p=0.1399$).

Upon segregation along the longitudinal axis of the hippocampus, there was no effect of JS ($F(1, 20) = 0.1436$, $p=0.7087$) nor a stress x biological sex interaction ($F(1, 20) = 0.09278$, $p=0.7638$) on the number of distal dendritic crossings per neuron in the dorsal hippocampus. Although it appeared that the number of number of distal dendritic crossings per neuron were lower in females compared to males, this effect

did not quite reach statistical significance ($F(1, 20) = 3.123$, $p=0.0924$; Figure 3.8E) and thus *post hoc* analysis could not be conducted.

In the ventral hippocampus (Figure 3.8F), there was no significant effect of stress ($F(1, 20) = 0.3087$, $p=0.5846$) nor a stress x biological sex interaction ($F(1, 20) = 0.3508$, $p=0.5603$) on distal dendritic crossings per neuron. The effect of biological sex did not quite reach statistical significance ($F(1, 20) = 3.767$, $p=0.0665$) and thus *post hoc* analysis could not be conducted.

This analysis shows that adult female rats have reduced distal crossings per DCX-positive cell in the hippocampus compared to adult males.

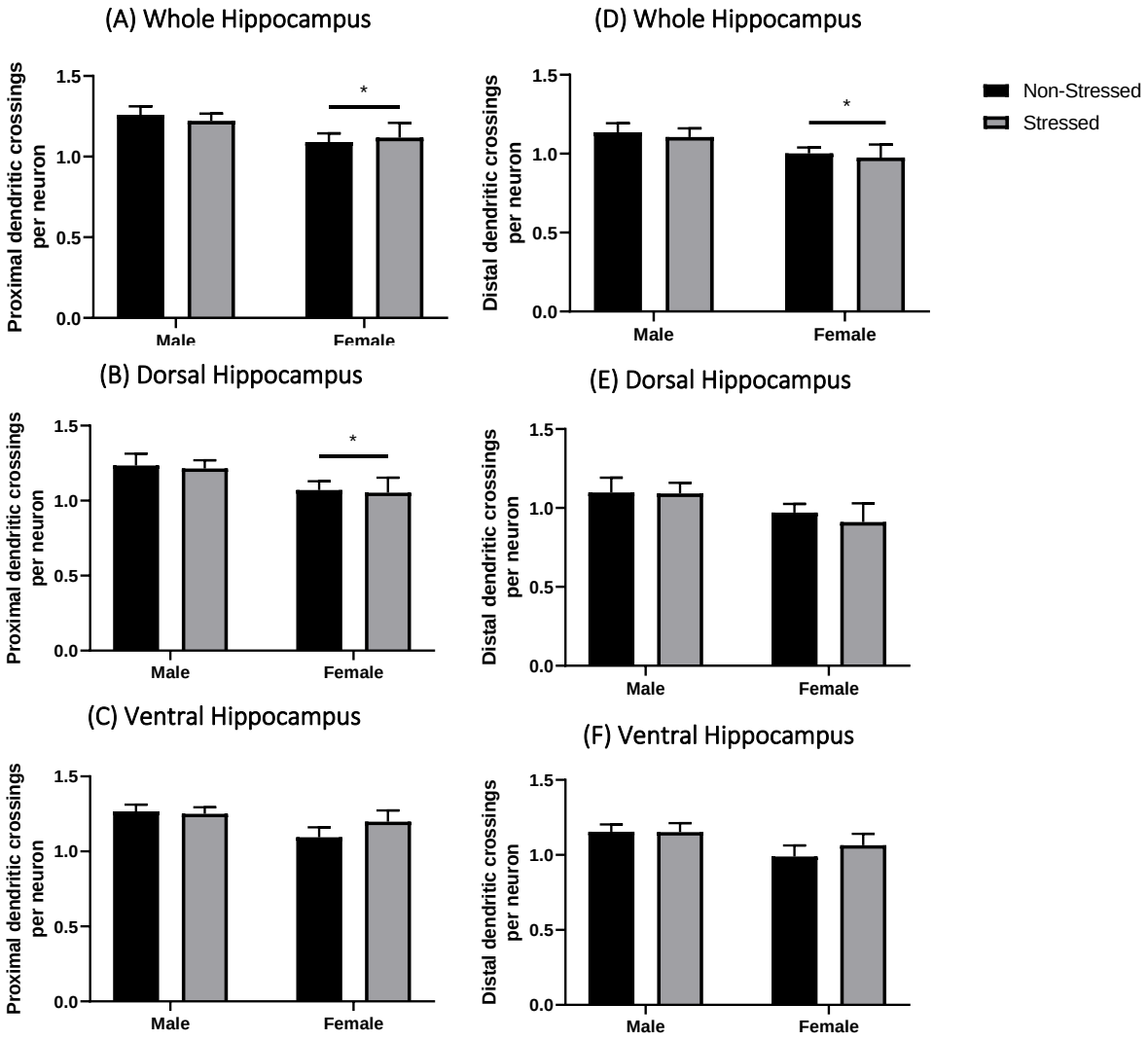


Figure 3.8: Proximal dendritic crossings per newly born neuron are lower in females than males but are not affected by juvenile stress in either male or female adult rats. The number of proximal dendritic crossings per neuron from new born hippocampal neurons are lower in females in the (A) whole and (B) dorsal hippocampus but not in the (C) ventral hippocampus. Distal dendritic crossings per newly born neuron are lower in females but are not effected by juvenile stress in either male or female adult rats. The number of distal dendritic crossings per neuron from new born hippocampal neurons are lower in females in the (D) whole hippocampus but not the (E) dorsal or (F) ventral hippocampus. Data are shown as mean +/- SEM. n = 6. *Main effect of biological sex, $p<0.05$.

3.4 The effects of juvenile stress on hippocampal microglia in adult male and female rats

To investigate the effects of JS on microglia in the dorsal and ventral hippocampus of male and female adult rats the number of microglia and their cell soma size were analysed.

3.4.1 In non-stressed rats, the number of microglia is lower in females than males in the dorsal but not ventral dentate gyrus of the hippocampus

To assess the effects of JS on microglia number in adult male and female rats, the number of IBA-1 positive cells was counted in the DG of the hippocampus (Figure 3.9).

Two-way ANOVA revealed that there was no effect of JS ($F(1, 20) = 0.009016$, $p=0.9253$) nor a stress x biological sex interaction ($F(1, 20) = 1.180$, $p=0.2902$) on the number of microglia in the DG of the hippocampus. However, there was a significant effect of biological sex whereby females exhibited lower numbers of IBA-1-positive cells compared to males ($F(1, 20) = 4.875$, $p=0.0391$; Figure 3.9A). Subsequent *post hoc* analysis revealed that non-stressed females had significantly fewer hippocampal microglia than non-stressed males ($p<0.05$) but no difference was found between stressed males and stressed females ($p=0.437$).

Upon segregation along the longitudinal axis of the hippocampus, there was no significant effect of JS ($F(1, 20) = 0.3160$, $p=0.5803$) nor a stress x biological sex interaction ($F(1, 20) = 2.031$, $p=0.1696$) on the number of microglia in the DG of the dorsal hippocampus (Figure 3.9B). However, there was a significant effect of

biological sex whereby females exhibited lower numbers of microglia compared to males ($F(1, 20) = 5.255$, $p=0.0329$) in the DG of the dorsal hippocampus. Subsequent *post hoc* analysis revealed that non-stressed females had significantly fewer hippocampal microglia than non-stressed males ($p<0.05$) but no difference was found between stressed males and stressed females ($p=0.5465$).

In the ventral hippocampus (Figure 3.9C), there was no effect of stress ($F(1, 20) = 0.1130$, $p=0.7402$), biological sex ($F(1, 20) = 1.139$, $p=0.2985$) nor a stress x biological sex interaction ($F(1, 20) = 0.002138$, $p=0.9636$) on the number of microglia in the DG.

This analysis shows that non-stressed adult female rats have a reduced number of microglia in the dorsal, but not ventral, hippocampus compared to adult non-stressed males.

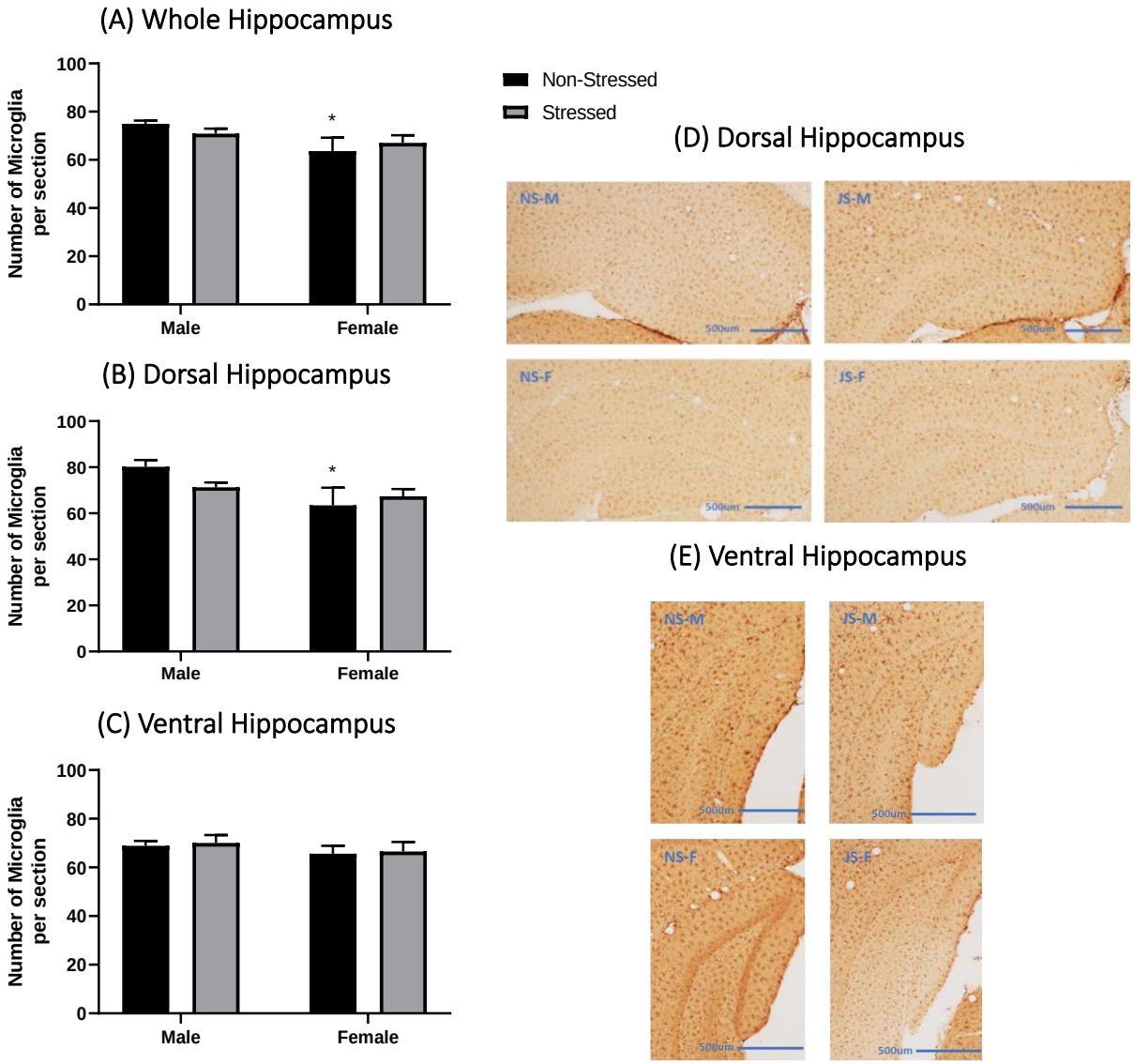


Figure 3.9: The number of microglia is lower in females than males in the dorsal (B) but not whole (A) or ventral (B) hippocampus without juvenile across the (A) whole, (B) dorsal and (C) ventral hippocampus. Representative images of the (D) dorsal and (E) ventral hippocampus stained with DCX are shown. Data are shown as mean +/- SEM. n = 6. DCX, Doublecortin; JS: Juvenile stress; NS: Non-Stressed; M: Male; F: Female; GCL: Granule cell layer; ML: Molecular layer. *Non-stressed males vs Non stressed females, $p<0.05$.

3.4.2 There is no effect of biological sex or stress on the number of microglia in the GCL of the hippocampus

To further characterise the effects of JS on microglia number in the dorsal and ventral hippocampus of adult male and female rats we counted the number of microglia in the GCL, a subregion of the DG, of the hippocampus (Figure 3.10).

A Kruskal-Wallis test revealed that there was no effect of JS, biological sex nor a stress x biological sex interaction ($H(3)=0.86$, $p=08351$) on the number of microglia in the GCL within the DG of the hippocampus (Figure 3.10A).

When the hippocampus was segregated into the dorsal and ventral hippocampus a similar effects was observed. In the GCL within the DG of the dorsal hippocampus (Figure 3.10B), there was no significant effect of stress, biological sex nor a stress x biological sex interaction on the number of microglia ($H(3)=5.051$, $p=0.1681$). In the GCL within the DG of the ventral hippocampus (Figure 3.10C), a two-way ANOVA revealed no significant effect of stress ($F(1, 20) = 0.1828$, $p=0.6736$), biological sex ($F(1, 20) = 0.01781$, $p=0.8952$) nor a stress x biological sex interaction ($F(1, 20) = 0.009154$, $p=0.9247$) on the number of microglia.

This analysis shows that there is no effect of stress or biological sex on the number of microglia in the GCL in the hippocampus of adult male or female rats.

3.4.3 The number of microglia in the molecular layer of the hippocampus is lower in females but is not affected by juvenile stress in either male or female rats.

To further characterise the effects of JS on microglia number in the dorsal and ventral hippocampus of adult male and female rats the number of microglia in the ML, a subregion of the DG, was counted (Figure 3.10).

A Kruskal-Wallis test revealed that there was a significant effect of JS, biological sex and a stress x biological sex interaction ($H(3)=12.62$, $p=0.0055$) on the number of microglia in the hippocampus (Figure 3.10D). Subsequent *post hoc* analysis revealed that non-stressed females have less microglia in the ML of the dorsal hippocampus than non-stressed males ($p<0.005$) and stressed females have less microglia in the ML of the dorsal hippocampus than stressed males ($p<0.05$).

When the ML of the hippocampus was segregated into the dorsal and ventral hippocampus (Figure 3.10E), there was no significant effect of stress ($F(1, 20) = 0.5344$, $p=0.4732$) nor a stress x biological sex interaction ($F(1, 20) = 1.134$, $p=0.2997$) on the number of microglia following a two-way ANOVA. However, there was a significant effect of biological sex whereby females exhibited lower numbers of IBA-1-positive cells compared to males ($F(1, 20) = 9.690$, $p=0.0055$; Figure 3.10E). Subsequent *post hoc* analysis revealed that non-stressed females had less microglia in the ML of the dorsal hippocampus than non-stressed males ($p<0.001$) but no difference in the number of microglia in the ML of the dorsal hippocampus was found between stressed males and stressed females ($p=0.163$).

In the ML of the ventral hippocampus (Figure 3.10F), there was no significant effect of stress ($F(1, 20) = 0.003818$, $p=0.9513$) nor a stress x biological sex interaction ($F(1, 20) = 0.1220$, $p=0.7305$) on the number of microglia. However, there was a significant effect of biological sex whereby females exhibited lower numbers of IBA-1-positive cells compared to males ($F(1, 20) = 10.60$, $p=0.0040$). Subsequent *post hoc* analysis revealed that stressed females had fewer microglia in the ML of the ventral hippocampus than stressed males ($p<0.05$) and the difference between non-stressed males and non-stressed females did not reach statistical significance ($p=0.0531$).

This analysis shows that adult non-stressed female rats have a reduced number of microglia in the ML in the dorsal, but not ventral, hippocampus compared to adult non-stressed males. It also shows that adult stressed female rats have a reduced number of microglia in the ML in the dorsal, but not ventral, hippocampus compared to adult stressed males.

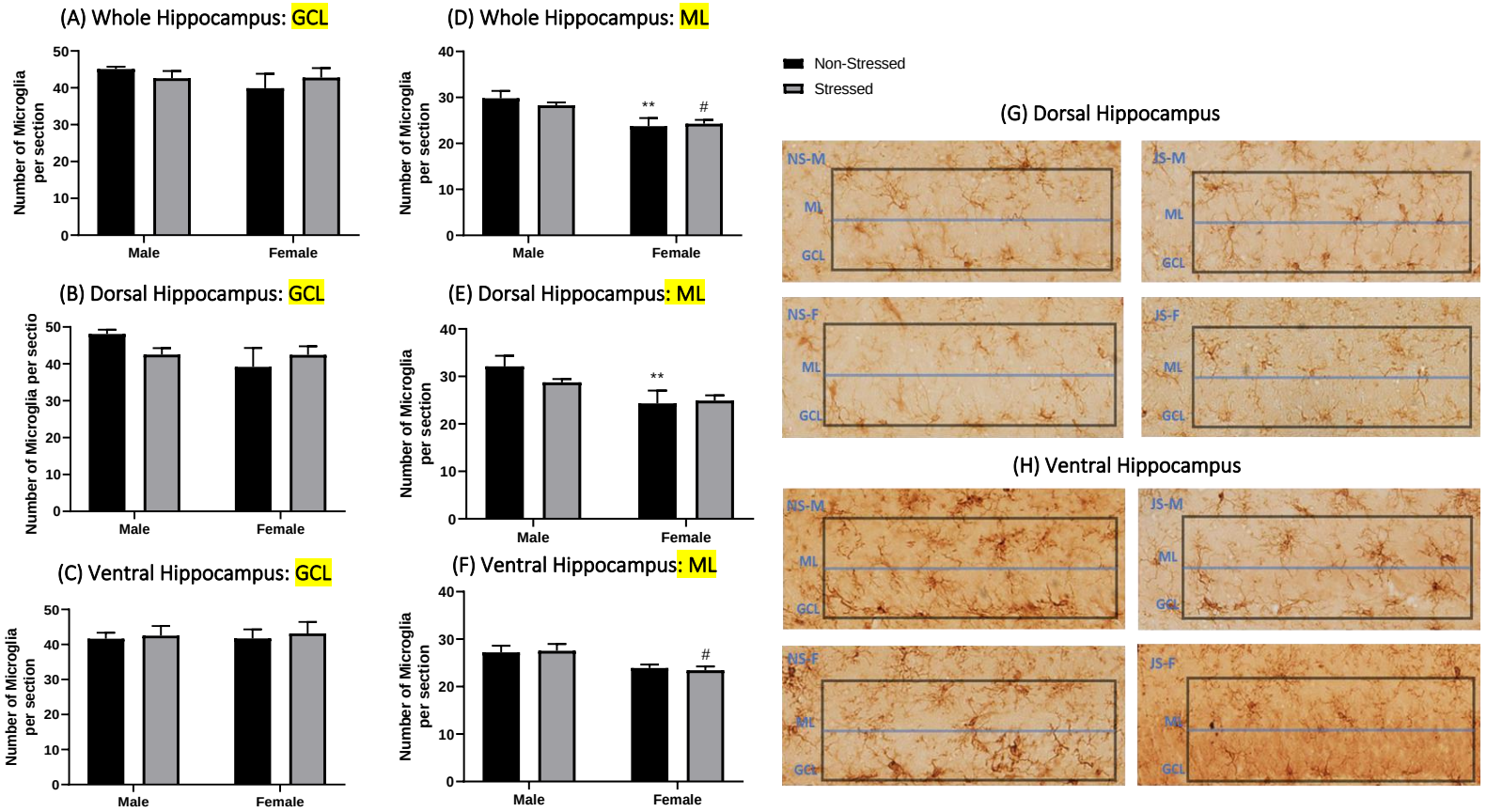


Figure 3.10: There is no effect of juvenile stress nor biological sex on the number of microglia in the GCL of the (A) whole, (B) dorsal or (C) ventral hippocampus. Females present with less microglia than males in the ML of adult rats. Non-stressed females have significantly less microglia than non-stressed males in the ML layer of the (D) whole and (E) dorsal hippocampus but not in the (F) ventral hippocampus. However, stressed females have significantly less microglia than stressed males across the (D) whole and (F) ventral hippocampus. Representative images depicting microglia in the GCL and ML or the (G) dorsal and (H) ventral hippocampus. Data are shown as mean +/- SEM. n = 6. JS: Juvenile stress; NS: Non-Separated; M: Male; F: Female; GCL: Granule cell layer; ML: Molecular layer. **Non-stressed males vs Non stressed females, p<0.01, #Stressed males vs stressed females, p<0.05.

3.4.4 There is no effect of juvenile stress or biological sex on the cell soma size of microglia in the DG of the hippocampus

To assess the effects of JS on microglial activation in the dorsal and ventral hippocampus of adult male and female rats, we measured the cell soma size of microglia in the DG of the hippocampus (Figure 3.11).

Two-way ANOVA revealed that there was no effect of JS $F(1, 20) = 0.01779$, $p=0.8952$), biological sex ($F(1, 20) = 0.3867$, $p=0.5411$) nor a stress x biological sex interaction ($F(1, 20) = 0.7021$, $p=0.4120$) on the cell soma size of microglia in the DG of the whole hippocampus (Figure 3.11A).

When the hippocampus was segregated into the dorsal and ventral hippocampus similar effects were observed. In the dorsal hippocampus (Figure 3.11B), there was no significant effect of stress ($F(1, 20) = 0.198$, $p=0.6611$), biological sex ($F(1, 20) = 2.334$, $p=0.1422$) nor a stress x biological sex interaction ($F(1, 20) = 1.361$, $p=0.2571$) on the cell soma size of microglia in the DG. In the ventral hippocampus (Figure 3.11C), there was no significant effect of stress ($F(1, 20) = 3.323e-007$, $p=0.99950$), biological sex ($F(1, 20) = 1.183$, $p=0.2897$) nor a stress x biological sex interaction ($F(1, 20) = 0.3769$, $p=0.5462$) on the cell soma size of microglia in the DG.

This analysis shows that there is no effect of stress or biological stress on the cell soma size of microglia in the hippocampus of adult rats.

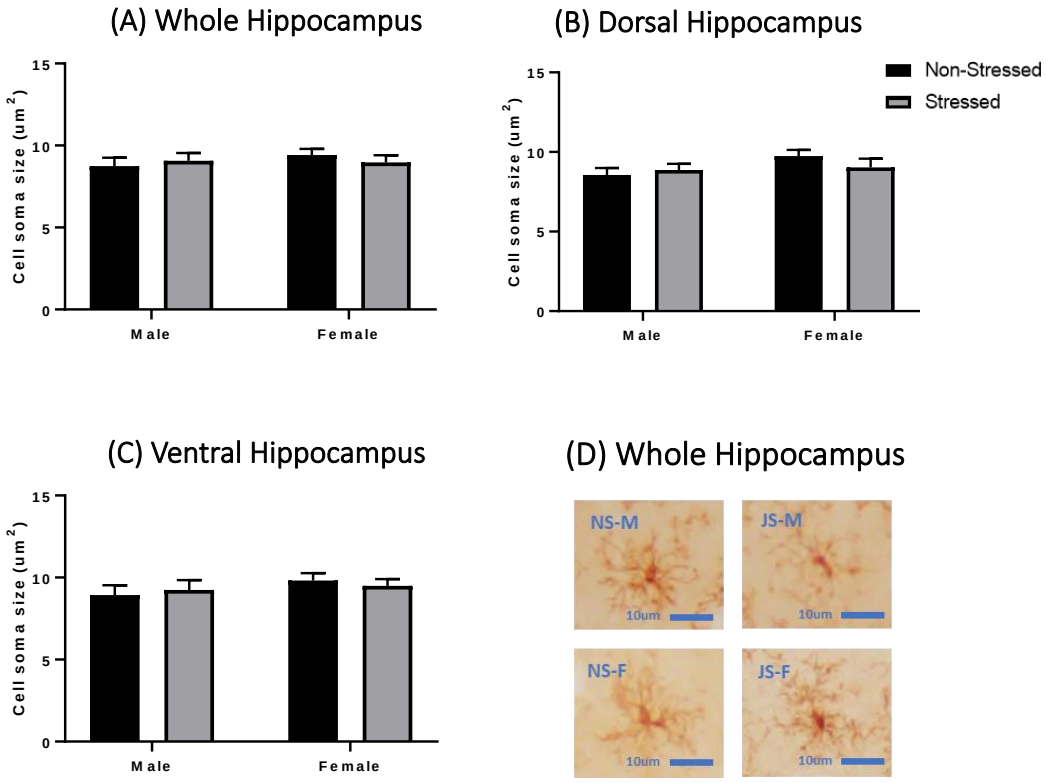


Figure 3.11: There is no effect of juvenile stress or sex on the cell soma size of microglia across the (A) whole, (B) dorsal or (C) ventral hippocampus. (D) Representative images depicting microglia soma size. Data are shown as mean +/- SEM. n = 6.

3.4.5 Females have larger microglia cell soma size than males in the GCL of the dorsal hippocampus and juvenile stress decreases cell soma size of microglia in the GCL of the dorsal hippocampus in adult female but not male rats

The data in sections 3.5.2 and 3.5.3 suggests that there were varied sub regional effects of JS and sex on microglia in the hippocampus. To further explore this, the cell soma size of microglia in the GCL and ML in the DG within the hippocampus were analysed (Figure 3.12).

Two-way ANOVA revealed that there was no effect of JS ($F(1, 20) = 0.3769$, $p=0.5462$), biological sex ($F(1, 20) = 2.183$, $p=0.1551$) nor a stress x biological sex interaction ($F(1, 20) = 2.904$, $p=0.1038$; Figure 3.12A) on the cell soma size of microglia in the GCL in the DG within the hippocampus. In the ML there was no effect of JS ($F(1, 20) = 0.003429$, $p=0.9539$), biological sex ($F(1, 20) = 1.524$, $p=0.2313$) nor a stress x biological sex interaction ($F(1, 20) = 0.01465$, $p=0.9049$) on the cell soma size of microglia in the ML in the DG within the hippocampus (Figure 3.12B).

When the hippocampus was segregated into the dorsal and ventral hippocampus there was no significant effect of stress ($F(1, 20) = 1.429$, $p=0.2459$) or biological sex ($F(1, 20) = 1.834$, $p=0.1908$) on the cell soma size of microglia in the GCL in the DG within the dorsal hippocampus. A significant stress x biological sex interaction ($F(1, 20) = 6.363$, $p=0.0202$) was revealed on the cell soma size of microglia in the GCL in the DG within the dorsal hippocampus (Figure 3.12C). Subsequent *post hoc* analysis revealed non-stressed females microglia cell soma size is larger than in non-stressed males ($p<0.05$). It also found that JS reduced microglia cell soma size in females (NS

females vs stressed females ($p<0.05$)) but not males. This effect on microglia cell soma size was not present in the ML in the DG within the dorsal hippocampus with stress ($F (1, 20) = 0.009348$, $p=0.9239$), biological sex ($F (1, 20) = 2.025$, $p=0.1702$) nor a stress x biological sex interaction ($F (1, 20) = 0.01071$, $p=0.9186$) revealing a significant effect (Figure 3.12D).

In the GCL in the DG within the ventral hippocampus there was no significant effect of stress ($F (1, 20) = 0.01411$, $p=0.9066$), biological sex ($F (1, 20) = 1.676$, $p=0.2102$) nor a stress x biological sex interaction ($F (1, 20) = 0.7674$, $p=0.3914$) on the cell soma size of microglia (Figure 3.12E). In the ML of the ventral hippocampus there was no significant effect of stress ($F (1, 20) = 0.03379$, $p=0.8560$), biological sex ($F (1, 20) = 0.4895$, $p=0.4922$) nor a stress x biological sex interaction ($F (1, 20) = 0.01048$, $p=0.9195$) on the cell soma size of microglia (Figure 3.12F).

This analysis shows that the cell soma size of microglia in the GCL in the dorsal hippocampus are increased in adult non-stressed female rats compared to males. However, the soma size of microglia in the GCL in the dorsal hippocampus of adult stressed female rats is reduced compared to non-stressed females.

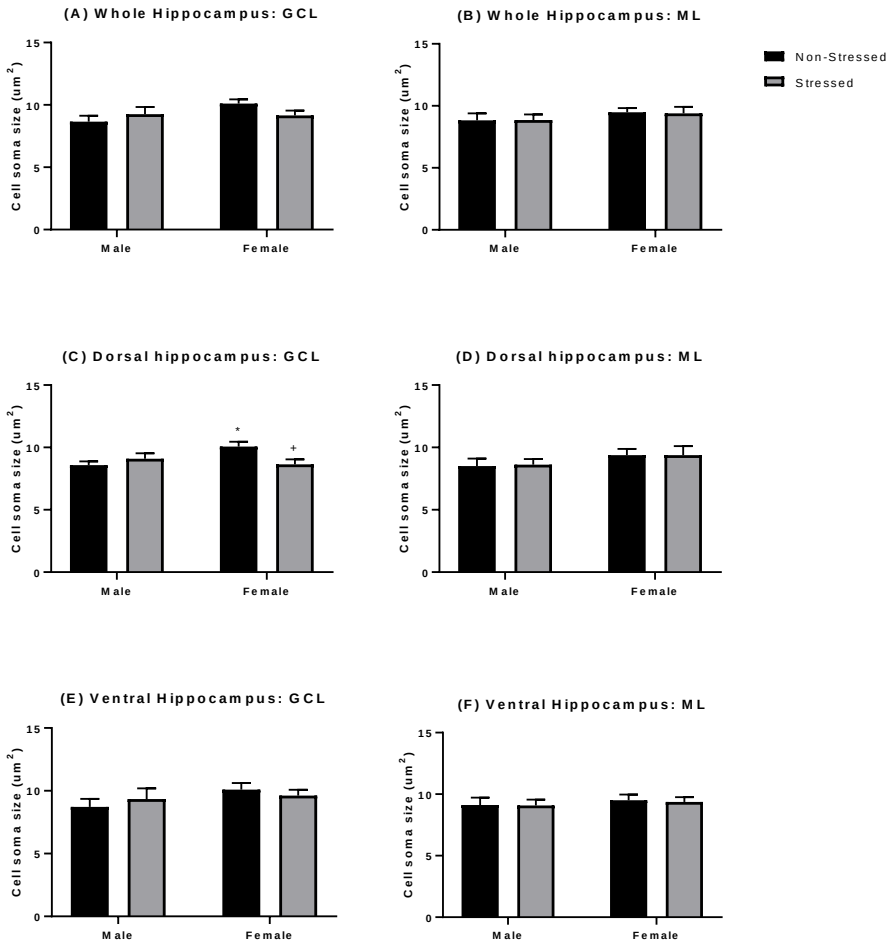


Figure 3.12. There is a significant interaction between biological sex and stress in the GCL of the dorsal hippocampus on the cell soma size of microglia. There was no effect of juvenile stress or sex on microglia cell soma size in the (A) GCL and (B) ML of the hippocampus. In the dorsal hippocampus microglia cell soma size was larger in non-stressed females than non-stressed males in the (C) GCL and microglia soma size of females is reduced with juvenile stress. There was no effect of sex or juvenile stress in the (D) ML of the dorsal hippocampus on cell soma size. There was also no effect of sex or stress on microglia soma size in the (E) GCL and (F) ML in the ventral hippocampus. Data are shown as mean +/- SEM. n = 6. GCL: Granule cell layer; ML: Molecular cell layer. *Non-stressed males vs Non stressed females, $p<0.05$. +Non-stressed females vs stressed females, $p<0.05$.

4. Discussion

Understanding the influence of psychological stress and inflammation on the brain during early life periods such as the juvenile period or adolescence is critical to identifying how these stressors are a risk factor for the development of stress-related psychiatric disorders (Davis *et al.* 2017). Over the past decade, evidence that adult hippocampal neurogenesis and microglia may play a role in the development of stress-related psychiatric disorders in response to stress during the perinatal and juvenile period has grown substantially (Agorastos *et al.* 2019; Frank *et al.* 2019; Youssef *et al.* 2019; Catale *et al.* 2020). However, research examining the immediate effects of psychological and immunological stressors on adult hippocampal neurogenesis and microglia is sparse. In fact, research to date has predominantly been conducted in male and not female rodents, even though females are more likely than males to develop stress-related psychiatric disorders (Bangasser and Valentino 2014). As such, we aimed to investigate the effects of MS stress and an acute inflammatory stressor, LPS, in juvenile female rats on hippocampal neurogenesis and neighbouring microglia, and to investigate the effect of JS on adult hippocampal neurogenesis and neighbouring microglia in male and female rats in adulthood, since measures of dendritic complexity of neurons are associated with function, whilst aberrant dendritic complexity has been associated with stress-related psychiatric disorders (Forrest *et al.* 2018; Pawley *et al.* 2020). We also measured the number of dendritic crossings of these newly born DCX+ neurons in the GCL (proximal crossings) and within the inner third of the ML (distal crossings).

In summary (see Table 4.1 also), we show that MS increased the number of newly born hippocampal neurons in the ventral hippocampus, reduced both proximal and distal dendritic crossings in the dorsal and ventral hippocampus, and increased the cell soma size of DG microglia, an indicator of activation. LPS alone had no effect of the number of newly born hippocampal neurons but it reduced proximal and distal dendritic crossings, and increased the number of microglia in the ML of the dorsal hippocampus, as well as the cell soma size of microglia in both the dorsal and ventral hippocampus in juvenile female rats (Table 4.1). LPS administration in MS increased proximal dendritic crossings when compared to MS or LPS alone, and decreased the number of distal dendritic crossings in the ventral hippocampus compared to LPS alone. The number of microglia in the dorsal hippocampus following these sequential stressors were decreased. We found that MS counteracted the impact of LPS on cell soma size, but that LPS following MS had no effect of microglial cell soma size in comparison to the MS group. The results also show that although juvenile stress had no effect on hippocampal neurogenesis in adult female or adult male rats, adult female rats had fewer newly born neurons in the hippocampus, and these newly born neurons had fewer proximal dendritic crossings in the dorsal hippocampus and fewer distal dendritic crossings than adult male rats (Table 4.1). Non-stressed adult female rats had fewer microglia in the dorsal hippocampus than males, and these effects were observed in the ML but not GCL. Finally, non-stressed adult females had microglia with an increased soma size compared to non-stressed males.

Table 4.1: The effects of (A) MS and LPS and (B) JS and Sex on hippocampal neurogenesis and colocated microlgia. [1]vs NS-SAL, [2]vs MS-SAL, [3]vs NS-LPS, [4]vs Males, [5]vs NS-M, and [6]vs NS-F. NS: Non-Separated/Stressed; MS: Maternal separation; SAL: Saline ; LPS: Lipopolysacharide; M: Male; JS: Juvenile Stress; F: Female; vHipp:Ventral Hippocampus; dHipp: Dorsal Hippocampus.

	Granule cell layer				**Molecular layer**		
	Hippocampal Neurogenesis		**Collocated Microglia**		**Hippocampal Neurogenesis**	**Collocated Microglia**	
	Number of DCX+ cells	**Proximal dendritic crossings**	**Number of IBA-1+ cells**	**Soma size**	**Distal dendritic crossings**	**Number of IBA-1+ cells**	**Soma size**
MS	vHipp: ↑[1]	↓[1]	-	↑[1]	↓[1]	-	↑[1]
NS-LPS	-	↓[1]	-	↑↑[1]	↓↓[1]	dHipp: ↑[1]	↑↑[1]
MS-LPS	-	vHipp:↑[2, 3]	-	↓[3]	vHipp: ↓[3]	dHipp: ↓↓[2, 3]	↓[3]
NS-M	-	-	-	-	-	-	-
JS-M	-	-	-	-	-	-	-
NS-F	↓[4]	dHipp: ↓[4]	-	dHipp: ↑[5]	↓[4]	↓[4]	-
JS-F	↓[4]	dHipp: ↓[4]	-	dHipp: ↓[6]	↓[4]	↓[4]	-

4.1 LPS administration in maternally separated juvenile female rats

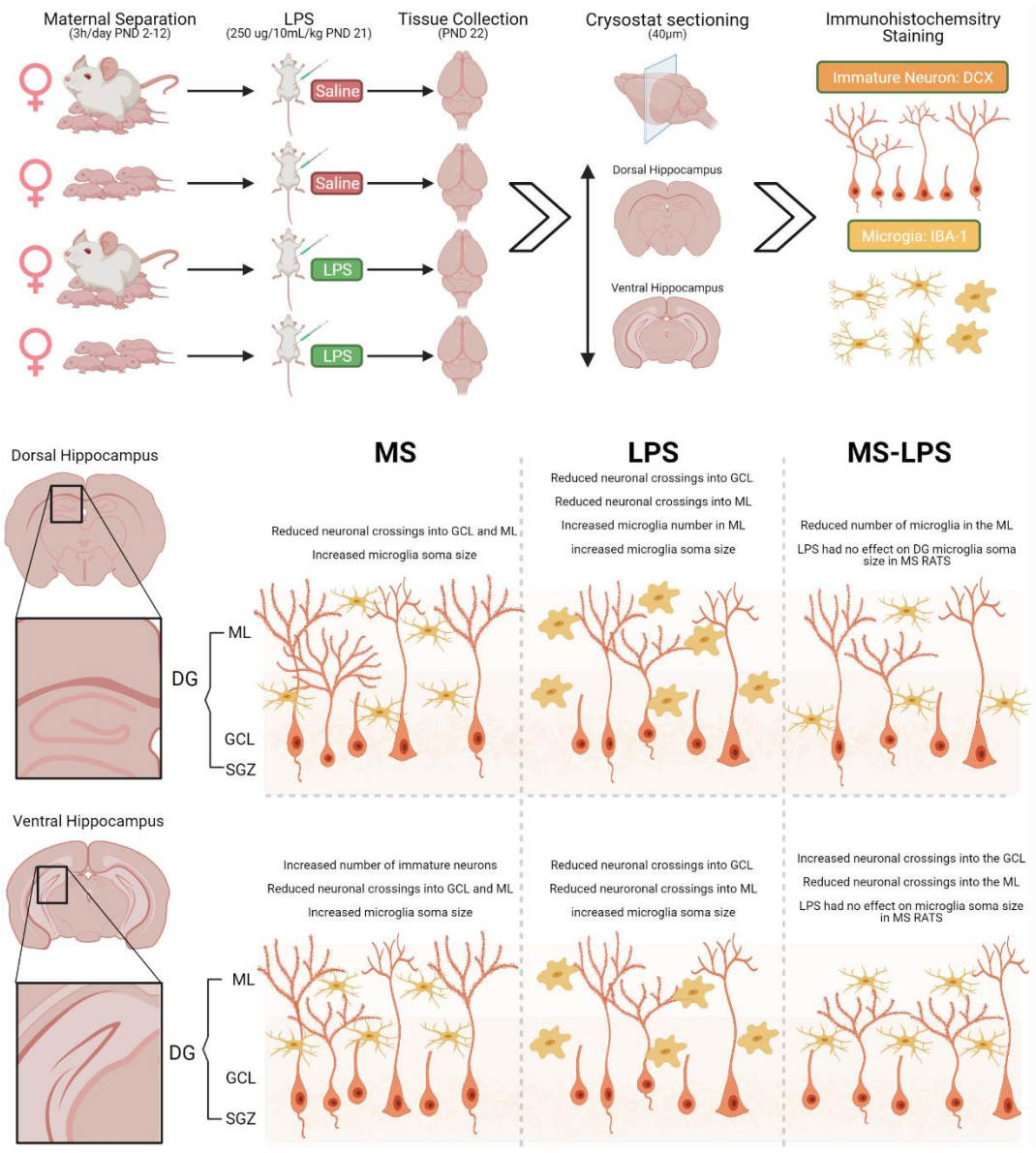


Figure 4.1: Summary of the effects of LPS administration in MS in adolescent female rats on hippocampal neurogenesis and microglial activation. Created with BioRender.com. DCX: Doublecortin; DG: Dentate gyrus; GCL: Granule cell layer; IBA-1: Ionized calcium-binding adapter molecule 1; LPS: Lipopolysaccharide; ML: Molecular layer; MS: Maternal Separation; PND: Postnatal day; SGZ: Subgranular zone.

4.1.1 The effects of maternal separation and LPS on adult hippocampal neurogenesis in juvenile female rats

We found that MS increased the number of newly born hippocampal neurons in the ventral hippocampus of juvenile female rats. Interestingly, it has been reported in juvenile male rats that MS from PND 1-21 for 3 hours each day decreased the number of mature adult born hippocampal neurons as measured by NeuN-positive immunohistochemistry (Oreland *et al.* 2010). Other studies examining the immediate effects of early life stress reveal varying results. Oomen *et al.,* (2009) found that a 24 hour MS on PND 3 of juvenile female rats significantly decreased the number of newly born hippocampal neurons as measured by DCX-positive immunohistochemistry on PND21 (Oomen *et al.* 2009), whilst our study found that a 3 hour MS regime from PND 2-12 had no effect on hippocampal neurogenesis in female rats when assessed on PND 21. In the study by Oomen *et al., (*2009), MS was completed on PND 3 which is when the neuroarchitecture of the GCL forms in the DG and thus if disrupted it might be detrimental to hippocampal neurogenesis throughout the remainder of the lifespan (Altman and Bayer 1990; Oomen *et al.* 2009). The greater stress of a 24 hour separation during DG development on PND 3 in comparison to the repeated 3 hour separations between PND 2-12 that we employed may explain the dramatic decrease in hippocampal neurogenesis observed by Oomen *et al.* (2009) in juvenile female rats compared to the lack of change in hippocampal neurogenesis observed in the current study. Multiple studies have reported that MS for 3 and 6 hours a day during the first 2 weeks of life resulted in decreased hippocampal neurogenesis in juvenile male rats (Oreland *et al.* 2010; Baek *et al.* 2011; Lajud 2012b; Lajud and Torner 2015). This suggests that different early

life stress paradigms; MS for 24 hours on PND 3 and MS for 3 hours daily between PND 2-12, may have different impacts on hippocampal neurogenesis in juvenile male and female rodents (Oreland *et al.* 2010; Baek *et al.* 2011; Lajud and Torner 2015). Therefore, it might be suggested that the discrepancy between results in the current study on hippocampal neurogenesis in adolescence may be explained by differences in the magnitude and timing of the stressor during early life as well as sex differences.

MS reduced the number of proximal and distal dendritic crossings of newly born hippocampal neurons in juvenile female rats. Chronic unpredictable stress reduces dendritic complexity of mature adult born hippocampal neurons in adult male mice, which may subsequently impair adult hippocampal neurogenesis associated functions (Dioli *et al.* 2019). However, to our knowledge there is only one study examining the dendritic morphology of newly born hippocampal neurons following MS for which show a reduction in the dendritic complexity of mature adult born hippocampal neurons in adult male and female mice, are in agreement with our data. (Leslie *et al.* 2011). Leslie *et al.,* (2011) observed that the dendritic complexity of mature adult hippocampal neurons measured by NeuN-positive immunohistochemistry was reduced following MS for 3 hours a day between PND 1-14 when they measured dendrite intersections at least 60µm from the soma of the mature adult born neuron in adult male and female mice. This is similar to our findings where the number of distal crossings (100µm from the cell soma) of newly born hippocampal neurons was reduced following MS for 3 hours a day between PND 1-14 in juvenile female rats much more than the number of proximal crossings (50

µm from the cell soma; Leslie *et al.* 2011). Leslie (2011) did not observe any sex differences in response to MS on dendritic complexity of mature adult born hippocampal neurons. However, recent evidence from Yagi *et al.* (2020) shows that there are transient sex differences in the dendritic development of adult male and female newly born neurons in the hippocampus of rats such that the dendrites of male newly born neurons are more mature after 2 weeks of age than those in female rats (Yagi *et al.* 2020). It is possible that the maturation and dendritic development of newly born hippocampal neurons following MS is sex dependent. Our study reported reduced dendritic complexity of newly born hippocampal neurons in the ventral hippocampus, which is associated with anxious or depressive behaviours, yet a study using the same MS paradigm in female rats as we did found that female rats are resistant to developing depressive and anxious behaviours following MS in adulthood (Hill *et al.* 2015; Dimatelis *et al.* 2016; Baptista and Andrade 2018). Juvenile female rats may produce depressive and anxious behaviours following MS for 3 hours a day between PND 2-12 that are no longer present in adulthood further demonstrating the importance of the timing of measurement of the effects of early life stress in these studies (Banqueri *et al.* 2017). More studies are needed to characterise the effects of stress in females, especially in early life, to understand the potential effects on neuronal morphology, dendritic retraction and associated functions particularly in adolescence which is when female bias in prevalence of stress-related psychiatric disorders begins to emerge.

LPS had no effect on the number of newly born neurons in the hippocampus of juvenile female rats. This was somewhat surprising given that inflammation has been reported to have a negative effect on newly born hippocampal neurons whether it is provoked prenatally, early in life or in adulthood (Green and Nolan 2014; Ryan and Nolan 2016; Perez-Dominguez *et al.* 2019). As NPCs differentiate into newly born hippocampal neurons they express the TLR4 receptor, a receptor for LPS (Rolls *et al.* 2007). TLR4 activation is associated with the release of proinflammatory cytokines, altered neuronal differentiation, dendritic remodelling, synaptogenesis and hippocampal neurogenesis dependent functions (Rolls *et al.* 2007; Okun *et al.* 2012; Shen *et al.* 2016; Chen *et al.* 2019). A study observing the effects of LPS on newly born hippocampal neurons found that LPS administration on PND 9 in male mice decreased the number of newly born hippocampal neurons in the dorsal but not ventral hippocampus on PND 60 (Järlestedt *et al.* 2013). Thus, the current result was surprising considering that it has predominantly been shown that inflammation decreases the number of newly born hippocampal neurons when induced in early life or adulthood (Green and Nolan, 2014; Ryan and Nolan, 2016). There is some evidence of a sexual dimorphism in response to inflammatory insults on hippocampal neurogenesis (Järlestedt *et al.* 2013; Valero *et al.* 2014; Conrad *et al.* 2015; Perez-Dominguez *et al.* 2019; Pawley *et al.* 2020), however, to date most studies on the impact of inflammation on adult hippocampal neurogenesis have either been conducted in males or have not considered sex differences. The adult hippocampal neurogenesis of the juvenile female rats in the current studies may have been protected from the negative impact of glucocorticoids by a protective interaction

with the female hypothalamic pituitary gonadal axis and the potential prepubertal release of estradiol (Green and McCormick 2016).

Although the number of newly born hippocampal neurons in female juvenile rats was not affected by LPS in our study, we did observe that LPS significantly decreased their dendritic complexity. Dinel *et al.,* (2014) has shown that 3 hours post-LPS is sufficient to detect a peripheral increase in proinflammatory cytokines, including IL-1β, in PND 14 male mice (Dinel *et al.* 2014). Pawley *et al.,* (2020) have recently shown that IL-1β overexpression decreases hippocampal neurogenesis and dendritic complexity of newly born hippocampal neurons. Dinel et. al., (2014) found that inflammation induced on PND 14 by LPS increased depressive and anxiety behaviours in male mice measured on PND 30, which might be mediated by an LPS-induced decrease in dendritic morphology of newly born hippocampal neurons similar to that observed in the current study. Conrad et. al., (2015) has shown that inflammation induced using a porcine reproductive and respiratory syndrome on PND 7 decreased the dendritic complexity of mature adult born hippocampal neurons measured using NeuN immunohistochemistry in PND 28 in both male and female piglets. This suggests that there may not be sex differences in the impact of inflammatory stress on hippocampal neurogenesis but there was a lack of similarity between the studies in the species examined (pig and rat), age of inflammatory stress (PND 7 and PND 21), type of inflammatory stress (viral and bacterial) and age of measure (PND 28 and PND 22). Together, further studies are warranted in juvenile female mice and rats.

Inflammation induced by LPS administration in juvenile female rats that had been previously exposed to MS did not exacerbate any MS or LPS-induced changes in hippocampal neurogenesis. Specifically, there was no effect of LPS following MS on the number of newly born neurons in the hippocampus, the MS-induced reduction in proximal dendritic crossings was attenuated by LPS; and while the distal dendritic crossings of newly born neurons were significantly reduced by LPS following MS although this effect was not greater than MS alone or LPS alone. Further, the attenuated effect of MS on LPS administration on proximal crossings, and the decrease in distal crossings following LPS following MS, was only significant in the ventral hippocampus. These were unexpected findings given that both stress and inflammation have previously been shown to negatively impact hippocampal neurogenesis in the DG, albeit predominantly in adult male rodents (Schoenfeld and Gould 2012; Ryan and Nolan 2016). Similarly, work such as that by Hennesy *et al.,* (2007) has found that MS provided a predisposition for greater pro-inflammatory responses to an inflammatory insult in male and female adult guinea pigs (Hennessy *et al.* 2007; Perkeybile *et al.* 2009). Other studies have identified that MS results in increased inflammation profile and increases the immunological response following LPS challenge measured in blood plasma and the cerebral cortex of adult male and female mice (Hohmann *et al.* 2017). However, our study did not observe MS exacerbating the effects of LPS on hippocampal neurogenesis in juvenile female rats.

The response to MS or other forms of psychological stress has previously been shown to have a greater negative impact on newly born neurons in the ventral hippocampus

than the dorsal hippocampus, which is more sensitive to stress and glucocorticoids (Levone *et al.* 2017). Whilst the impact of inflammatory insults on hippocampal neurogenesis are not specific to either the dorsal or ventral hippocampus (Green and Nolan 2014). Therefore, it was somewhat unexpected to find that MS increased neurogenesis in the ventral hippocampus. To the best of my knowledge, there is no study which employed the MS protocol and analysed adolescent hippocampal neurogenesis at PND 22 in male or female rats, thus it is possible that the observed increase in the number of neurons in the ventral hippocampus might be a sex specific difference although future studies are required to directly test this hypothesis. It is interesting that whilst MS alone and LPS alone reduced dendritic crossings in the GCL, LPS following MS increased the number of proximal crossings in the ventral hippocampus compared to MS alone and LPS alone. However, distal crossings into the ML were further reduced by LPS following MS in the ventral hippocampus but not in the dorsal hippocampus. Our group have recently shown that long term exposure of corticosterone to neurospheres isolated from the dorsal and ventral hippocampus of PND 28 male rats reduce dendritic complexity, especially in neurospheres isolated from the ventral hippocampus (Levone *et al.* 2020). This might be due to the higher sensitivity of newly born neurons to stress (and stress hormones) in the ventral hippocampus (Onufriev *et al.* 2018; Levone *et al.* 2020). LPS has previously been shown to induce higher levels of corticosterone in the ventral hippocampus than the dorsal hippocampus of adult male rats (Onufriev *et al.* 2018). The ventral hippocampus therefore, may be more sensitive to the effects of LPS on adult hippocampal neurogenesis due to the potential mediating effect of corticosterone (Levone *et al.* 2017; Anacker *et al.* 2018; Levone *et al.* 2020).

4.1.2 The effects of maternal separation and LPS on hippocampal microglia in juvenile female rats

There was no effect of MS on the number of microglia in the DG of the hippocampus in juvenile female rats, when examined across the whole hippocampus, GCL and ML subregions, nor in the dorsal or ventral hippocampus. Delpech *et al.,* (2016) found that MS of male mice for 3 hours a day from PND 1-14 increased the number of microglia in the DG of the hippocampus on PND14 which was not present in PND 28 male mice that underwent a MS for 3 hours a day from PND 1-21 (Delpech *et al.* 2016). Another group found that MS of male rats for 4 hours a day from PND 1-21 had no effect on the number of microglia in the DG or hilus subregions of the hippocampus of adult male rats (Banqueri *et al.* 2019). Interestingly, they observed that MS increased the number of microglia in the CA3 in adulthood (Banqueri *et al.* 2019). MS of male rats for 3 hours a day from PND 1-10 has been shown to increase the number of microglia in the hippocampus (subregion not specified) when measured on PND 10, 20 and 30 but not on PND 40 or 60 (Réus *et al.* 2019). In contrast, we observe that MS did not effect the number of microglia in DG of the hippocampus in PND 22 female rats. Torner and colleagues (Roque *et al.* 2016; Saavedra *et al.* 2017) have shown that MS of male rats for 3 hours a day from PND 1-14 did not affect the number of microglia in the hilus of the hippocampus on PND 15, yet in a later study by this group found that the same MS procedure decreased the number of microglia in the hilus and CA3 of the hippocampus of PND 15 male rats. Therefore, the effect of MS on the number of microglia varies dependent on the

length of the MS, the subregion of the hippocampus being analysed, the sex of the animal and the age at which the microglial population is analysed. Although to the best of my knowledge, there has been no work investigating the effect of MS on the microglial population in the DG in female rodents, it is possible that juvenile female rats might have a protective mechanism that males do not have which might explain the discrepancy between our findings and those in the literature on males. However, MS did increase the cell soma size of microglia across the DG of juvenile female rats. In agreement, it has been previously reported that MS of male rats for 3 hours a day from PND 1-14 increased the activation status of hippocampal microglia as assessed by morphological changes when observed on PND 15 (Roque *et al.* 2016). Similarly, Delpech *et al.,* (2016) found that MS of male mice from PND 1-14 induced a peripheral proinflammatory immune response and increased the phagocytic activity of hippocampal microglia, which is associated with an activated morphology, when measured on PND 14 (Delpech *et al.* 2016). Interestingly in the same study, MS of male mice for 3 hours a day from PND 1-21 did not increase the phagocytic activity of hippocampal microglia or produce a peripheral pro-inflammatory response when measured on PND 28 (Delpech *et al.* 2016). This suggests that the age of analysis of microglia following early life stress or the length of the MS paradigm may be significant factors which could influence findings. Furthermore, our study included an 8 day recovery period between the end of MS and cull on PND 22 which may account for our differing findings. During the postnatal period there are transient sex differences in microglial development: there is more IBA-1 expression on PND 4 in female rats than in male rats, and this difference is no longer apparent on PND 5 (Osborne *et al.* 2019). Therefore, MS during this time period may disrupt this

transient increase in microglia density in the PND 4 female rat hippocampus and produce sex dependent effects on microglia detectable in the hippocampus of PND 22 female rats. Much of the research previously discussed measuring the effects of MS on microglia in the hippocampus has not focused on the DG and has been in male rodents. It is possible that microglia responsivity to MS in the subregions in the hippocampus of male and female rodents is different so further research is necessary.

We did not find any change in the number of microglia in the GCL of the hippocampus following LPS, whilst LPS was found to increase the number of microglia in the ML of the dorsal hippocampus. While the number of microglia in the DG was found to be increased in response to LPS in adult male mice (Furube *et al.* 2018), and LPS treatment in adult male rats has previously been found to increase the number of microglia in the GCL (Lana *et al.* 2017). There are no studies that we are aware of reporting changes in the microglial population in the ML alone following LPS challenge, nor in juvenile female rats. Microglia adjacent to the neurogenic niche are responsive to changes in the environment and contribute to homeostasis of the microenvironment of the neurogenic niche (Diaz-Aparicio *et al.* 2020). A contributing factor to our location dependent findings of the impact of LPS on microglia in the GCL and ML of the hippocampus may be microglial regional heterogeneity, which refers to the location dependent density, morphology and ontogenesis (Furube *et al.* 2018; Tan *et al.* 2020). There is also evidence that the dorsal hippocampus produces a greater pro-inflammatory response to LPS than the ventral hippocampus (Onufriev

et al. 2018), which may contribute to the fact that LPS increased the number of microglia observed in the ML of the dorsal and not ventral hippocampus of juvenile female rats following LPS administration in the current study. We found that the cell soma size of microglia was increased (an index of phagocytosis and thus activation status) in the DG 24 hours after LPS administration in juvenile female rats. This has been previously shown in adult male and female mice (Savage *et al.* 2019). There is evidence that there could be age and sex dependent effects of LPS on microglia. It is established that there are sex differences in neuroinflammation in aged males and females, where aged females have been shown to be more responsive to neuroinflammatory stress than aged males (Nelson and Lenz 2017; Murtaj *et al.* 2019). A study by Osborne *et al.,* (2019) found sex and region specific microglial reactivity to neonatal infection (*E.coli*) on PND4 in the hippocampus of PND 4 male and female rat pups. Microglia in PND 4 male rats were more likely to present with a thin morphology than females, while microglia in the female PND 4 hippocampus presented with a stout morphology which is associated with immature or activated microglia, in response to E.coli infection (Osborne *et al.* 2019). However, more research on these sex differences in response to inflammation during early life and adolescence is needed.

When LPS was administered to maternally separated juvenile rats we found that the number of microglia in the ML of the dorsal hippocampus was reduced compared to LPS or MS alone, an effect that we did not observe with either MS alone or LPS alone. A previous study in male juvenile rats found that MS for 3 hours a day from PND 1-

14 followed by LPS on PND 14 and sacrifice on PND 15 reduced the number of microglia in the hilus and CA3 regions of the hippocampus of juvenile male rats (Saavedra *et al.* 2017). However, Saavedra *et al.,* (2017) study also showed that MS irrespective of LPS administration brought about this reduction of microglia in the hippocampus (hilus and CA3), which we did not find in the GCL or ML of the DG of the hippocampus. These effects may be due to the fact that we have analysed the number of microglia in the ML and GCL of the DG and not the CA3 or hilus. There is evidence that inflammation in early life stress and adolescence may produce sex-dependent neuroinflammatory effects. Neonatal LPS-stress can increase cyclooxygenase-2 expression in the hypothalamus in adult male but not female rats (Kentner *et al.* 2010), and mixed modality stress between PND 37-48 exacerbates the inflammatory response to LPS in the hippocampus of adult male but not female rats (Pyter *et al.* 2013). The differences we are finding may be differences in response to MS in male and female juvenile rats. Alternatively, the difference of neuroinflammatory response to MS and LPS in our study in comparison to the findings of Saavedra *et al.,* (2017) may be due to the difference in the ages of animals at which analysis of the number of microglia took place (PND 14 vs PND 22) between the two studies. In fact, Saavedra *et al.,* (2017) have shown that the age of analysis has an impact on the outcome of neuroinflammatory challenge on hippocampal microglia (Saavedra *et al.* 2017). We also found that LPS failed to further increase the cell soma size of microglia in the DG of MS juvenile female pups. Neonatal stress by restraint stress of pregnant mice can exacerbate microglia response to LPS, increasing expression of proinflammatory cytokines such as IL-6 and TNF-α and increasing the density of IBA-1 positive cells measured using immunohistochemistry

compared to LPS alone in adult female mice (Diz-Chaves *et al.* 2012). Interestingly, Saavedra *et al.,* (2017) found that LPS administration in MS had no effect on cell soma size in the CA3 but increased the microglia soma size in the hilus compared to LPS alone and MS alone in PND 14 male rats.

In contrast to the sensitivity of newly born neurons in the ventral hippocampus to stress, it appeared that microglia in the dorsal hippocampus were more sensitive to early life stress and LPS than microglia in the ventral hippocampus of juvenile female rats. Sub-regional differences in the number of microglia between the GCL and ML were evident. LPS following MS reduced the number of microglia in the ML of the dorsal hippocampus compared to MS alone and LPS alone and LPS had no effect on the soma size of microglia in MS juvenile female rats. It appears that microglia in the ML of the dorsal hippocampus were differently affected by MS and LPS when compared to microglia in the GCL or subregions of the ventral hippocampus. It has been previously shown that immunotoxins increase microglia associated gene expression in the dorsal but not ventral hippocampus (Dobryakova *et al.* 2018). Early life stress has been shown to reprogram microglial responses to future inflammatory interventions: it may be the case that here this alteration in response to LPS is in part specific or at least stronger in microglia within the ML of the dorsal hippocampus which may be due to differences in cytokine and corticosterone concentrations in the dorsal and ventral hippocampus following an inflammatory stress (Onufriev *et al.* 2018) and the influence of the neurogenic niche on microglial activity in the granule cell layer (Battista *et al.* 2006). MS and LPS impacted microglia and dendritic complexity differently in the GCL and ML: the number of microglia in the GCL was not affected in the hippocampus following MS and LPS, whilst dendritic development in

the GCL of the ventral hippocampus increased by MS and LPS when compared to MS alone or LPS alone. In the ML, we found that LPS decreased the dendritic development into the ML in the whole hippocampus and increased the number of microglia in the dorsal hippocampus. LPS alone produced a greater state of microglial activation than MS alone or MS and LPS in the whole hippocampus. It may be that this raised state of activation was the primary factor which determined the reduction in ML dendritic development. However, following MS and LPS, the number of microglia in the dorsal hippocampus was significantly reduced, whilst the dendritic development into the ML was further reduced in the ventral but not dorsal hippocampus compared to LPS alone. The significant reduction in dendritic development the ML of the ventral hippocampus may be because there are less microglia which could contribute to dendritic remodelling of newly born hippocampal neurons (Wu *et al.* 2015).

The differences between the GCL and ML microglia and their effects on hippocampal neurogenesis may be due to the neurogenic niche present in the SGZ adjacent to the GCL. Microglia are extremely responsive to their environment and since the neurogenic niche is highly regulated, microglia function can be regulated here (Battista *et al.* 2006; Bonafina *et al.* 2020). The neurogenic niche in the SGZ could influence microglia in the GCL to act as guardians of hippocampal neurogenesis and dendritic development (Battista *et al.* 2006; Gomes-Leal 2012). Microglia in the ML may alternatively contribute to reducing dendritic development. It has been previously shown that the increased expression of inflammatory cytokines and

glucocorticoids induced by LPS differ in the dorsal hippocampus, which accumulates more pro-inflammatory cytokines, and the ventral hippocampus, which accumulates more glucocorticoids in rodents (Onufriev *et al.* 2018). This may explain the difference in microglial responses to LPS preceded by MS in the dorsal and ventral hippocampus.

4.2 Juvenile stress in adult male and female rats

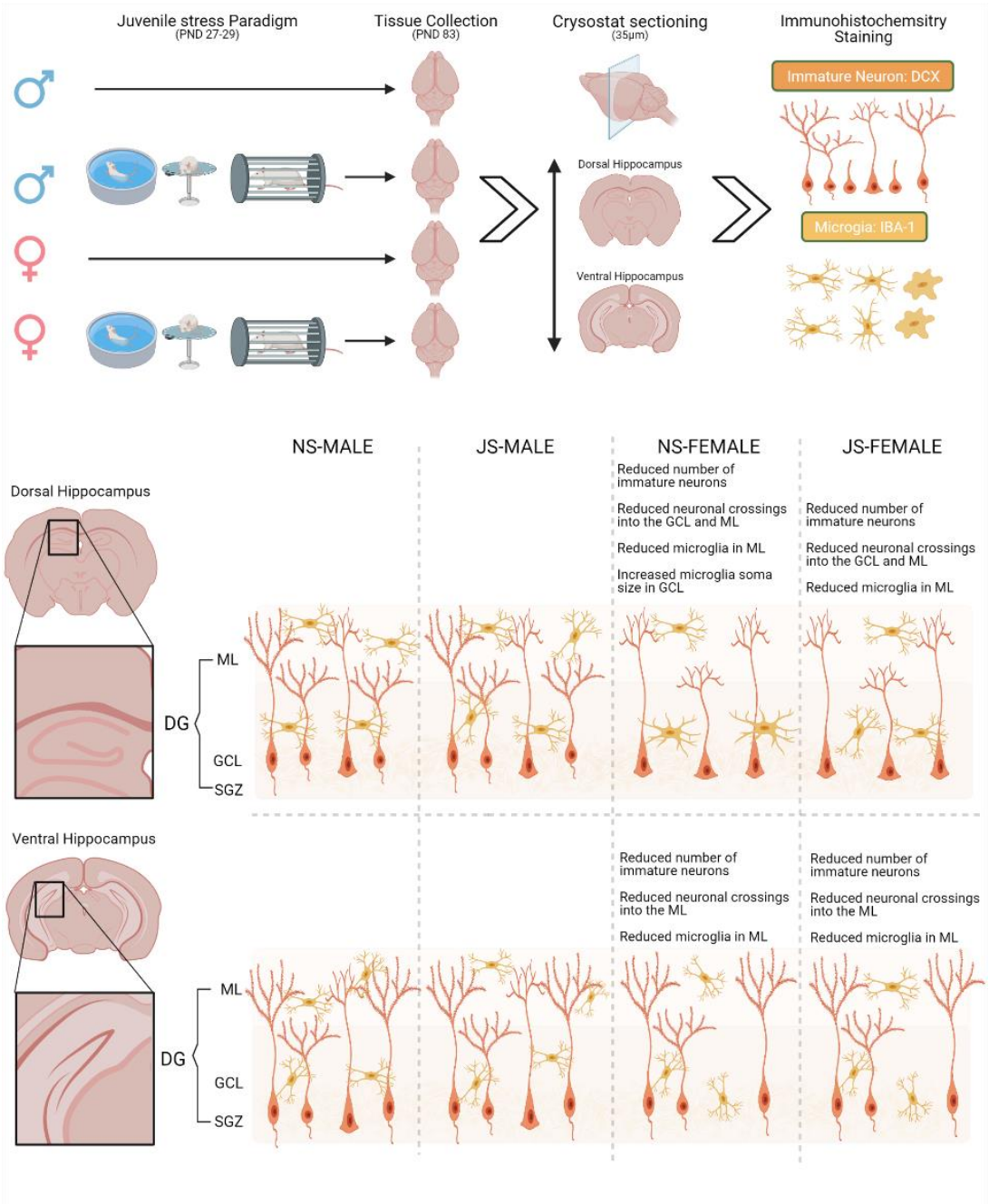


Figure 4.2: Summary of the effects of JS in adult male and female rats on hippocampal neurogenesis and microglial activation. Created with BioRender.com. DCX: Doublecortin; DG: Dentate gyrus; GCL: Granule cell layer; IBA-1: Ionized calcium-binding adapter molecule 1; JS: Juvenile Stress; LPS: Lipopolysaccharide; ML: Molecular layer; PND: Postnatal day; SGZ: Subgranular zone.

4.2.1 The effects of juvenile stress and biological sex on hippocampal neurogenesis in adult male and female rats

We found that JS applied in PND 27-29 had no effect on the number of newly born hippocampal neurons or proximal and distal dendritic crossings in either adult male and female rats. It has been well established that stress during early life decreases hippocampal neurogenesis in adulthood making this is an unexpected result (Karten *et al.* 2005; Oomen *et al.* 2010; Loi *et al.* 2014; Lajud and Torner 2015). A study using JS between PND 25-27 found that the number of newly born hippocampal neurons as measured by DCX-positive immunohistochemistry in the ventral hippocampus almost doubled in male but not in female rats aged between PND 60-65 following JS (Brydges *et al.* 2018). In agreement with our findings, Bryges *et al.,* (2018) reported that JS did not alter the number of newly born hippocampal neurons in adult females (Brydges *et al.* 2018). In this study, the number of DCX cells/μm^2 was analysed in hippocampi from rats aged between PND 60-65 whilst we analysed our DCX cells/section in hippocampi from rats on PND 83. The importance of the age of the animal when analysis is carried out is significant, as it may be that the 3 weeks of age between histological analysis in the current study and the findings by Brydges *et al.,* (2018) could explain the observed difference in hippocampal neurogenesis in male rats. This study also observed that JS altered pattern separation and fear conditioning (dorsal and ventral hippocampal neurogenesis associated tasks) when measured in adult male but not female rats (Brydges *et al.* 2018). Therefore, we expected to observe dendritic remodelling of newly born hippocampal neurons, but we did not find that to be the case. Richter-Levin and Zitman (2013) have shown that JS (acute swim stress) increased the excitability of neurons measured using *in vivo*

electrophysiology in the DG of adult male and female rats. However, they observed that this effect was more pronounced in adult male rats with only male rats responding with increased intrinsic excitability and frequency dependent inhibition (Zitman and Richter-Levin 2013). Interestingly, Segal and colleagues observed that the Richter-Levin *et al.,* (2013) JS model between PND 27-30 in male rats has contrasting effects on synaptic plasticity in the dorsal and ventral hippocampus. The Richter-Levin and colleagues JS model decreased long-term potentiation in the dorsal hippocampus and increased long-term potentiation in the ventral hippocampus of adult male rats (Maggio and Segal 2011; Grigoryan *et al.* 2015). It may be that the Richter-Levin and colleagues model of JS may not alter the number of newly born hippocampal neurons or their dendritic development in the DG but instead hippocampal functional changes as observed by Brydges *et al.,* (2018) may occur via different mechanisms such as altered synaptic plasticity and neurotransmission in other regions of the hippocampus (such as the CA1: Zitman and Richter-Levin 2013; Grigoryan *et al.* 2015).

Although we did not see any impact of juvenile stress on the number or dendritic complexity of newly born hippocampal neurons in either sex in adulthood, previous studies have reported that juvenile stress can produce multiple long term and sex-dependent effects on the hippocampus and hippocampus-associated functions in adulthood. Brydges *et al.,* (2014) previously reported that JS increased anxiety related behaviour and altered the expression of GR and MR in male and female mice (Brydges *et al.* 2014a). This study found that anxiety behaviours were increased in

adult rats measured on PND 99 although this increase occurred to a lesser extent in females than males, and females had a greater expression of GR in the hippocampus than males (Brydges *et al.* 2014a). Another study from the same group found that JS impaired contextual fear in adult male but not female rats. They also found that JS enhanced performance in spatial navigation tasks in female but not male rats (Brydges *et al.* 2014b). Finally, JS has been shown to increase the number of GABergic interneurons in the ventral hippocampus and increase GAD67 (rate limiting enzyme for GABA) in the ventral hilus of male rats, thus it should be considered that this, rather than differences in adult hippocampal neurogenesis, might have contributed to the reported juvenile stress-induced changes in behaviour (Albrecht *et al.* 2017; Brydges *et al.* 2018).

A significant finding in this thesis was the observation that there are significant sex differences in adult hippocampal neurogenesis. We found that the number of newly born hippocampal neurons and their dendritic complexity is significantly lower in the adult female rat hippocampus than their male counterparts. This complements a finding by Hillerer *et al.,* (2013) showing that the basal number of newly born hippocampal neurons in adulthood is lower in female rats than males (Hillerer *et al.* 2013).

Similarly, it was also shown by Yagi *et al*., (2020) that there are significant sex differences in the maturation and attrition of newly born hippocampal neurons in adult rats where newly born hippocampal neurons mature faster and in greater

numbers in adult male compared to female rats (Yagi *et al.* 2020). Their data suggests that during the first 2 weeks of development of newly born hippocampal neurons (during which time DCX is expressed), more neurons are born and mature faster in males than females. There appears to be no significant differences in the number of mature adult born hippocampal neurons between adult male and female rats (Yagi *et al.* 2020). Therefore, the sex difference we observed in adult hippocampal neurogenesis between male and female adult rats is likely transient in nature. We can add to these findings that it appears that dendritic development during the first 2 weeks of newly born hippocampal neurons development may be lower in females. However, Yagi *et al.,* (2020) did not explore the dendritic complexity of newly born hippocampal neurons (Yagi *et al.* 2020).

We did not observe a difference in the number of newly born hippocampal neurons in the dorsal or ventral hippocampus in male and female rats. Yagi *et al.,* (2020) have also shown that adult female rats had more NSCs in the ventral than dorsal hippocampus and that adult male rats had more NSCs than females with no sub regional difference (Yagi *et al.* 2020). Thus, it would be expected that the ventral hippocampus of female rats would have a greater capacity for neurogenesis but this was not observed in this thesis. We found that there were less proximal dendritic crossings per newly born hippocampal neuron in the dorsal hippocampus of adult females compared to male rats. It could be that male rats have more proximal dendritic complexity in the dorsal hippocampus than females, and this may account for the sex differences in spatial navigation (Brydges *et al.* 2018). Males tend to

perform better than females in spatial navigation tasks in humans (Voyer *et al.* 2017) and rodents (Jonasson 2005) so it is possible that dorsal hippocampal neurogenesis associated functional impairment in females may be due to lower dendritic development per newly born neuron in the dorsal hippocampus.

4.2.2 The effects of juvenile stress and sex on hippocampal microglia in adult male and female rats

We found that JS had no effect on the number or soma size of microglia in the hippocampus of adult male rats. JS also did not alter the number of microglia in the hippocampus of adult female rats but it significantly reduced the cell soma size of microglia in the GCL of the female dorsal hippocampus compared to non-stressed females. This is in contrast to previous reports on the impact of stressors on microglial density and soma size. For instance, it has been reported that prenatal stress increased microglia number, increased the soma size and exacerbated the pro inflammatory response to LPS in the hippocampus of adult male rats and mice (Diz-Chaves *et al.* 2013; Ślusarczyk *et al.* 2015). Early life stress, such as MS, prior to adolescence has also been found to increase the number of microglia in the CA3 of the hippocampus and to increase the expression of markers of microglial motility and phagocytic activity in adult male rodents (Delpech *et al.* 2016; Banqueri *et al.* 2019; Réus *et al.* 2019). Chronic stress in adulthood has also been shown to increase the number of microglia and decrease the cell soma size of microglia in the CA3 of the hippocampus of adult male rats (Tynan *et al.* 2010). It has previously been shown that early social isolation stress between PND 14-21 reduced the number and

reduced the soma size of microglia in the DG of the hippocampus of adult male and female mice (Gong *et al.* 2018) and these findings in females mirror our finding that juvenile stress decreased soma size in female GCL dorsal hippocampal microglia, although we did not see any effect in males. However, similar to our study, social instability stress between PND 30-45 did not alter microglia number and morphology in juvenile male rats and in adult male rats (McCormick *et al.* 2012), whilst another study using social defeat stress between PND 28-37 found that the number of microglia in the prefrontal cortex of adult male mice was increased immediately following stress but decreased in adulthood suggesting a regional effect of stress during the juvenile period (Zhang *et al.* 2019a; Zhang *et al.* 2019b). In addition, it is also possible that the impact of juvenile stress on microglia may depend on the length of time the stressor was applied and the intensity of the stress. Indeed, in our study we used a short-term stress paradigm for 3 days and thus might not have been of sufficient intensity to induce long-lasting microglial changes that would persist into adulthood.

In the absence of JS, we found significant sex-dependent regional differences in microglial number and soma size in adult male and female rats. In the DG of the hippocampus, non-stressed adult female rats had a lower number of microglia than non-stressed adult male rats. When we explored sub regional differences, female and male rats had equal numbers of microglia in the GCL whilst females had significantly less microglia than males in the ML of the hippocampus. Guneykaya *et al.,* (2018) reported that microglia are present in a higher density in the hippocampus

(subregion not specified) of adult male than female mice which is in agreement with the findings of this thesis (Guneykaya *et al.* 2018). However, opposing findings have also been reported whereby it was reported that adult females have significantly more microglia than adult male rats in the DG of the hippocampus (Mouton *et al.* 2002; Schwarz *et al.* 2012). Extending on previous studies, we assessed sub-regional densities of microglia in the GCL and inner ML of the hippocampus and found sex differences in the ML but not the GCL of the dorsal and ventral hippocampus.

When we assessed microglial cell soma size, we found a sex difference in the GCL of the dorsal but not ventral hippocampal microglia soma size, whereby adult non-stressed females had a significantly larger cell soma sizes than adult non-stressed males. In adulthood and throughout ageing, female microglia numbers increase and undertake a more activated morphology than males (Mouton *et al.* 2002; Mangold *et al.* 2017). Schwartz *et al.,* (2012) have previously confirmed that microglia in the DG of adult female rats have a more activated morphology than males (Schwarz *et al.* 2012). However, other studies in mice have contradicted these findings (Guneykaya *et al.* 2018). These three studies assessed microglia in different hippocampal subregions (Mouton: DG and CA1; Schwartz: DG, CA1-3; Guneykaya: unspecified). Together, these findings show that sex differences are not uniform throughout the DG and that studying the subregions of the hippocampus may be crucial to develop insight into sex differences in microglia in the hippocampus.

Since microglia regulate adult hippocampal neurogenesis as measured by NSC proliferation, differentiation and dendritic modelling (Koss and Frick 2017; Yagi and

Galea 2019), it was of interest to determine whether differences in microglia density and soma size along the longitudinal axis might correlate with differences in neurogenesis along the longitudinal axis of the hippocampus. Indeed, studies have recently shown that corticosterone and cytokines following LPS stress, which affect microglia gene expression and morphology, accumulate differently in the dorsal and ventral hippocampus of male rats (Onufriev *et al.* 2018). Onufriev *et al.,* (2018) also showed that microglia in the dorsal hippocampus are more primed than ventral hippocampal microglia to respond to inflammatory insult in adult male rats (Onufriev *et al.* 2018). The expression of estrogen receptors is also greater in the dorsal than ventral hippocampus of female rats (Shughrue and Merchenthaler 2000), so the more pronounced responsivity of female microglia to stress in the dorsal hippocampus could be explained by differences in the responsivity of microglia, and astrocytes and neurons, to stress, inflammation and sex hormones (Pyter *et al.* 2013; Villa *et al.* 2016). Here we found that the microglia in the female GCL of the dorsal hippocampus had a significantly more activated morphology than males. While it may be tempting to speculate that this may have contributed to the reduced numbers of newly born neurons and proximal dendritic crossings in the hippocampus of females it is important to note that the female-associated reductions in newly born neurons and proximal dendritic crossings did not occur in the dorsal hippocampus alone. Thus, the sex differences in adult neurogenesis that we observed here do not seem to be correlated with microglial density and activity.

4.3 Limitations and Future Studies

The MS and LPS study in juvenile female rats characterised cell numbers in different regions of the hippocampus and the significance of some of our findings is limited by a low sample number (n=4). There is also no paper with the same timings of MS, LPS and age of analysis in male rodents as were carried out in this thesis, so although some of our findings may be sex specific, it cannot be concluded without a direct comparison of males to females within the same experiment. We did not analyse dendritic morphology and microglia in the outer molecular layer. A follow-up study could expand the number of frames measuring microglia and dendritic crossings into the outer ML. Microglia differences in the outer ML may influence synaptic transmission between newly born hippocampal neurons and hilar perforant path-associated cells in the outer ML to orchestrate hippocampal function (Acsady and Kali 2007; Raza *et al.* 2017). We were limited in our assessment as we did not detect the stage of the oestrus cycle and analyse our data relative to it in the adult female rats. Estrogen has been shown to regulate microglia morphology and expression which may interact with our analysis (Vegeto *et al.* 2006). Astrocytes also contribute to dendritic remodelling in the hippocampus and it would interesting to assess if there are sex differences in astroglia in the GCL and ML of the DG in the hippocampus (Mouton *et al.* 2002; Lana *et al.* 2017).

To better understand changes in the hippocampal neurogenesis and microglial population, injection and then staining of hippocampal sections for BrdU would have provided insight into the changes in proliferation of NSCs into newly born

hippocampal neurons and microglia. MS prevented microglia from becoming further activated by LPS, further work measuring an array of pro-inflammatory and anti-inflammatory cytokines could provide insight to our findings. I hypothesise that microglia in the GCL are more likely to undertake an alternatively activated state whilst microglia in the ML are more likely to become classically activated following early life stress (Lively and Schlichter 2018). To investigate this, co-staining CD-16/32 with IBA-1 for classical activation and CD206 with IBA-1 for alternatively activated microglia in the DG could be performed (Beier *et al.* 2017). Western blot or immunohistochemistry analysis for changes in protein expression associated with different states of microglial activation could reveal whether these microglia were in classically activated or alternatively activated states. Evidence of the contribution of astrocytes working alongside microglia and neurons in the DG to regulate neurogenesis, synaptogenesis and blood-brain-barrier permeability is growing (Lana *et al.* 2017). Future studies should consider co-staining using fluorescent immunohistochemistry for GFAP (astrocytes) as well as for microglia and newly born neurons. Looking into the effect of stressors on synapses, and if microglia and astrocytes are precisely collocated with synapses, could also provide further insight into the contribution of glia following MS or LPS to regulating function in juvenile female rats.

4.4 Conclusion

In conclusion, the results of this thesis have shown the effects of stress at different ages and type (MS/LPS/JS) on hippocampal neurogenesis and microglia. MS

differentially altered the response of adult hippocampal neurogenesis and microglia to further inflammatory intervention in the dorsal and ventral hippocampus, as well as within the GCL and ML providing evidence that early life stress can alter future inflammatory responses. JS did not induce any long-term effects on adult hippocampal neurogenesis or microglia in males but it reduced the cell soma size of female microglia. Adult female rats were found to have fewer newly born neurons and dendritic development than males, particularly in the dorsal hippocampus where we also observed significant sex differences in microglia cell number and morphology. This thesis has highlighted the importance of factors such as sex, brain structure subregion, the age at which stress is experienced and the age at which hippocampal neurogenesis and microglia are assessed when considering the effects of stress and inflammation in juvenile and adult male and female rats. However, further work is required to understand the mechanisms underpinning differences in newly born neuron development and microglia in specific subregions of the hippocampus between male and female rats, if they are related to sex differences in hippocampus associated function and the development of stress-related psychiatric disorders.

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Appendix

Table 1. Microglial polarization states and substances produced (Subramaniam and Federoff 2017).

Activation type/function	Source	Substances produced	Reference
M1 (classical activation): pro-inflammatory and pro-killing	LPS, IFN-γ	*Cytokines*: IL-1β, IL-6, IL-12, IL-17, IL-18, IL-23, TNF-α *Markers*: CD86, MHC-II *Chemokines*: CCL2 *Metabolic enzyme/redox molecules*: iNOS, COX-2, reactive oxygen species and reactive nitrogen species prostaglandin E2	Mahad and Ransohoff, 2003; Kawanokuchi et al., 2006 Kawanokuchi et al., 2008; Loane and Byrnes, 2010; Benarroch, 2013; Chhor et al., 2013; Franco and Fernandez-Suarez, 2015; Nakagawa and Chiba, 2015
M2a (alternative activation): tissue repair and phagocytosis	IL-4, IL-13	*Cytokines*: IL-10 *Markers*: CD206, SR-A1, SR-B1, Arg1, Ym1, Fizz1 *Others*: extracellular matrix proteins, PPAR	Mahad and Ransohoff, 2003; Loane and Byrnes, 2010, Benarroch, 2013; Chhor et al., 2013; Franco and Fernandez-Suarez, 2015; Nakagawa and Chiba, 2015
M2b (alternative activation): recruitment of regulatory T cells	Fcγ receptors, TLRs and immune complexes (IgG)	*Cytokines*: IL-1β, IL-6, IL-10, TNF-α *Markers*: CD86, MHC-II *Others*: SOCS3, COX-2, Sphk	
M2c (alternative activation): anti-inflammatory and healing	IL-10, TGF-β and glucocorticoids	*Cytokines*: IL-10, TGF-β *Markers*: CD163	

Arg1, arginase 1; CCL, chemokine (C-C motif) ligand; CD, cluster of differentiation; COX-2, cyclooxygenase-2; Fizz1, found in inflammatory zone; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; MHC-II, major histocompatibility complex II; SOCS3, suppressor of cytokine signaling-3; Sphk, sphingosine kinase; SR-A1, scavenger receptor class A1; SR-B1, scavenger receptor class B1; TGF, transforming growth factor; TNF-α, tumor necrosis factor-α; Ym1, chitinase-like protein.